Université de Sherbrooke

Involvement of the Jak/STAT pathway in platelet-activating factor receptor signaling

Viktoria Lukashova

Department of Pediatrics, Immunology Program

Thesis presented to the Faculty of medicine with a view to

obtain the grade of Doctor of Philosophy (Ph.D.) in Immunology,

Université de Sherbrooke, Sherbrooke, Québec, Canada.

15 January 2003

Your file Votre référence
ISBN: 0-612-85174-5
Our file Notre référence
ISBN: 0-612-85174-5

TABLE OF CONTENTS

ABBREVIATIONS

bFGF: basic fibroblast growth factor

cAMP: cyclic adenosine monophosphate

CAT: chloramphenicol acetyltransferase

CIS: cytokine-induced SH2-containing protein

DAG: diacylglycerol

EPO: Erythropoietin

EPOR: Erythropoietin receptor

ERK: extracellular signal-regulated kinase

GDP: Guanine diphosphate

GPCR: G-protein-coupled receptor

GTP: Guanine triphosphate

GH: Growth hormone

GHR: Growth hormone receptor

GM-CSF: Granulocyte macrophage colony-stimulating factor

hPAFR: Human platelet-activating factor receptor

HRE: hormone responsive element

IL: Interleukin

ILR: Interleukin receptor

IFN: Interferon

IFNR: Interferon receptor

IP: Inositol phosphate

Jak: Janus kinase

JAB: Jak binding protein

JH domain: Jak homology domain

LPS: lypopolysacharide

MAPK: mitogen-activated protein kinase

NLS: nuclear localization signal

PAF: Platelet-activating factor

PAFR: Platelet-activating factor receptor

PI3K: Phosphatidyl inositol 3-kinase

PIAS: Protein inhibitor of activated STAT

PLC: Phospholipase C

PMA: phorbol 12-myristate 13-acetate

PKC: Protein kinase C

PTX: Pertussis toxin

RA: retinoic acid

SHP: SH2-domain containing protein tyrosine phosphatase

SOCS: Suppressor of cytokine signaling

SSI: STAT induced STAT inhibitor

STAT: Signal transducer and activator of transcription

T_3: thyroid hormone

TPO: Thrombopoietin

TGF-β: transforming growth factor β

TNF-α: tumor necrosis factor α

RÉSUMÉ

Le facteur activateur de plaquettes (PAF) est un médiateur phospholipidique, exerçant son influence au niveau des systèmes nerveux, cardio-vasculaire, reproducteur, respiratoire et immunitaire. Le PAF est produit par une grande variété de cellules, incluant les monocytes/macrophages, neutrophiles, lymphocytes et cellules endothéliales. Ces cellules peuvent être à la fois source et cible du PAF.

Les effets du PAF passent par l'activation d'un récepteur spécifique faisant partie de la grande famille des récepteurs couplés aux protéines G (GPCRs). Une fois stimulés, les récepteurs du PAF peuvent activer des effecteurs variés: des canaux ioniques, des adénylate cyclases, des phospholipases (PLA_2, PLC, PLD) et certaines kinases (PKC, PI3K, MAPK). Il a été démontré que les inhibiteurs des protéines kinases inhibent l'activation de PLD, MAPK et PLA_2 induite par le PAF. La nature des kinases impliquées reste méconnue, cependant.

Dans cette étude, nous avons démontré que le PAF active des tyrosine kinases Jak2 et Tyk2 dans les cellules des lignées myéloïdes MonoMac-1 et U937, ainsi que dans les cellules COS-7 transfectées de façon transitoire avec le récepteur du PAF et les kinases. Il s'ensuit alors une phosphorylation des résidus tyrosines des facteurs STAT1, STAT2, STAT3 et STAT5 ainsi qu'une translocation des STAT1 et STAT3 au noyau. Nous avons trouvé que Tyk2 est associée avec le récepteur du PAF et qu'elle est nécessaire pour l'activation du promoteur du PAFR.

Pour étudier les mécanismes de la transcription du PAFR dependant de Tyk2, nous avons utilisé les récepteurs mutants non-couplés aux protéines G. Nous avons aussi créé des minigènes codant pour les boucles intracellulaires du PAFR et examiné leur

capacité à inhiber la signalisation. Nous avons trouvé que les récepteurs mutants D63N, D289A, Y293A, non-couplés aux protéines G, étaient capables d'induire l'activation du promoteur du PAFR via Tyk2. Nous avons déterminé que la deuxième boucle intracellulaire et la queue C-terminale du récepteur sont importantes pour la transcription du PAFR dependante de Tyk2. De plus, la substitution E51A dans la première boucle intracellulaire du PAFR abolit la capacité du récepteur à induire l'activation du promoteur du PAFR.

Nous avons aussi exploré les mécanismes de l'activation de Jak2 par PAFR. Nous avons trouvé que Jak2 est activée par le PAF indépendamment des protéines G, de l'internalisation et de la translocation des arrestines. L'association de Jak2 avec le récepteur a été détectée après la stimulation par le PAF et seulement en présence de Tyk2 active. L'activité catalytique de Tyk2 a aussi été impliquée dans l'activation de Jak2 après stimulation par le PAF. La co-expression d'un mutant dominant négatif de Tyk2, K930I, a aboli la phosphorylation de Jak2.

À l'aide des mutants de délétion de Tyk2 et du PAFR ainsi que des protéines de fusion, portant des boucles intracellulaires du PAFR, nous avons démontré que PAFR a des sites multiples de liaison de Tyk2.

En résumé, notre travail démontre que le récepteur du PAF induit la voie d'activation Jak/STAT et que cette voie joue un rôle dans la régulation transcriptionelle du promoteur du PAFR. En plus, nous avons défini les mécanismes d'activation de Tyk2 et Jak2 comme indépendants de protéine G, de l'internalisation et des arrestines. Nous avons déterminé les régions du récepteur importantes pour le couplage et l'activation de Tyk2.

ABSTRACT

Platelet-activating factor (PAF) is a potent phospholipid mediator involved in a variety of pathophysiological events, such as inflammation, asthma, cardiovascular disease, reproduction, and cerebral ischemia. PAF activates multiple signaling pathways and triggers a diverse array of biological actions by interacting with a specific receptor that belongs to the G-protein coupled receptor family (GPCR). Many reports suggested the involvement of tyrosine kinases in the activation of mitogen-activated protein kinase cascade and phospholipase Cγ, but the nature of the kinases as well as exact mechanisms have not been clarified.

Initially we were interested in determining whether PAF could activate the Jak/STAT pathway. This signaling cascade is known to be stimulated by cytokines and growth factors, however, recent reports indicate that it is also implicated in GPCR-mediated signal transduction. We found that PAF induced rapid Jak2 and Tyk2 tyrosine phosphorylation in the monocytic cell line MonoMac-1 and in COS-7 cells transiently transfected with PAFR and Tyk2 or Jak2 cDNAs. Tyk2 activation in MonoMac-1 cells was rapid and declined to basal levels after 5 min of PAF treatment, while Jak2 phosphorylation was sustained for a longer period. In COS-7 cells, transiently expressing PAFR and Jaks, Jak2 tyrosine phosphorylation was comparable to that in MonoMac-1 cells, whereas the increase in the level of tyrosine phosphorylation of Tyk2 was lower.

In MonoMac-1 cells, PAF-induced activation of kinases was followed by transient tyrosine phosphorylation of STAT1, 2, 3 and sustained phosphorylation of

STAT5 and subsequent STAT1 and STAT3 translocation to the nucleus. In a reconstituted system, PAF-mediated STAT1 and STAT3 nuclear translocation required the presence of Tyk2.

PAF is known to upregulate its own receptor in certain cells. To explore the role of the Jak/STAT pathway in regulation of PAFR transcription, we used a PAFR promoter 1 construct that consists of several putative STAT-binding sites. Our results showed that Tyk2 was obligatory for PAF-stimulated PAFR promoter 1 activation.

To study the mechanisms of Tyk2-dependent PAFR transcription we used mutants with impaired G-protein coupling and C-terminal deletion mutants of the receptor. We also created minigene constructs encoding the intracellular loops of PAFR and investigated their ability to inhibit signaling. We found that mutant receptors D63N, D289A, Y293A, which do not couple to G-proteins, were capable of inducing Tyk2-dependent PAFR promoter activation. We also determined that the PAFR second intracellular loop and the C-terminus of the receptor are important for Tyk2-dependent-PAFR transcription. The most surprising finding was the identification of a single point mutation E51A in the first intracellular loop, which resulted in a receptor incapable of mediating PAFR promoter 1 activation.

We also explored the mechanisms of Jak2 activation by PAFR. The G-protein-uncoupled mutant PAFR D289A induced Jak2 phosphorylation. The mutants of PAFR T305Stop, M311Stop, C317Stop and D289A, which do not induce arrestin translocation, nevertheless stimulated Jak2 phosphorylation. We observed inhibition of PAF-mediated Jak2 activation by co-transfection of a minigene encoding the C-terminus of PAFR.

Altogether, these results indicate that PAFR mediates Jak2 phosphorylation independently of G-protein activation, internalization and arrestin translocation.

Jak kinases are constitutively associated with cytokine receptors. In contrast, association between GPCRs and Jaks had been shown to be ligand-dependent, although small amounts of Jaks associated with certain GPCRs could be detected in quiescent cells. To study PAF receptor interaction with Janus kinases, we used COS-7 cells transiently transfected with receptor and kinase cDNAs. We found that Tyk2 co-immunoprecipitated and co-localized with PAFR, independently of agonist binding. Co-localization of the two proteins was also observed in MonoMac-1 cells treated, or not, with PAF. In addition, Tyk2 co-immunoprecipitated with the PAFR G-protein uncoupled mutants, D289A and Y293A, in the absence of PAF stimulation, confirming that the kinase is constitutively associated with the receptor. In contrast, Jak2 association with the receptor was detected after PAF stimulation and only in the presence of catalytically active Tyk2. Tyk2 catalytic activity was also involved in PAF-mediated Jak2 activation. Co-expression of a dominant-negative mutant Tyk2 K930I abolished PAF-stimulated Jak2 tyrosine phosphorylation, suggesting that Tyk2 transphosphorylates or facilitates Jak2 phosphorylation in response to ligand.

To determine which region of Tyk2 bound PAFR, we used truncated mutants of the kinase. We observed that each of the deletion proteins, containing the Jak homology regions JH6-7 (residues from 1 to 262), or JH3-5 (263-601), or entire N-terminus (1-601), can associate with PAFR. To further characterize the PAFR-Tyk2 interaction, we generated a series of receptor deletion mutants and GST-fusion proteins bearing different

intracellular loops of the receptor. Our results show that PAFR has multiple binding sites for Tyk2. GST-fusion proteins bearing the second and third intracellular loops as well as the C-terminus of the receptor bound full length Tyk2 and the truncated Tyk2 mutant (1-601), while GST-fusion protein encompassing the first intracellular loop was able to bind only the truncated kinase. The mutant receptor T305Stop immunoprecipitated Tyk2, indicating that the C-terminus, alone, is not essential for binding the kinase. Deletions in the second intracellular loop (Δ131-133, Δ127-133) of the receptor resulted in a partial decrease of Tyk2 binding.

In summary, our work demonstrates that the PAF receptor triggers the activation of the signaling cascade, Jak/STAT, and this may lead to the up-regulation of the receptor. In addition, we defined the mechanism of Tyk2 and Jak2 activation as G-protein- and arrestin-independent. We also determined the receptor regions important for Tyk2 binding and activation.

I. INTRODUCTION

PAF and its receptor

Platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine; PAF) is a potent phospholipid mediator that is involved in a wide range of biological responses, such as vasodilatation, contraction of smooth muscle, activation of neutrophils, macrophages, and eosinophils. PAF plays important roles in allergic disorders, inflammation, asthma, endotoxin shock, etc. (Izumi et al., 1995; Izumi and Shimizu, 1995; Chao and Olson, 1993). The terminology of platelet-activating factor was originally introduced by Benveniste and colleagues (Benveniste et. al., 1972) for the soluble substance released from basophils following IgE stimulation. Later, three independent laboratories described a new class of ether phospholipids with biological activities identical to PAF (Demopoulos et al., 1979; Blank et al., 1979; Benveniste et al, 1979).

PAF is synthesized by either a remodeling or a *de novo* pathway (Figure 1). While the remodeling route was activated during inflammation and other hypersensitivity responses, the *de novo* pathway was thought to be the source of PAF required for physiological functions (Snyder et al., 1996). The remodeling pathway requires the tightly coupled reaction of phospholipase A_2 and acetyl-CoA:lyso-PAF acetyltransferase. Phospholipase A_2 activation results in hydrolysis of membrane phospholipids to generate a variety of 2-lysophospholipides (lyso-PAF). These lysophospholipids are the substrates of acetyl-CoA: lyso-PAF acetyltransferase, which catalyzes the transfer of the acetyl moiety from acetyl-CoA to the free hydroxyl at the *sn*-2 position of the lysophospholipids. The *de novo* pathway involves the synthesis of 1-O-alkyl-

2acetylglycerol, which is then converted to PAF by a specific cholinephosphotransferase (Bussolino and Camussi, 1995, Peplow, 1999).

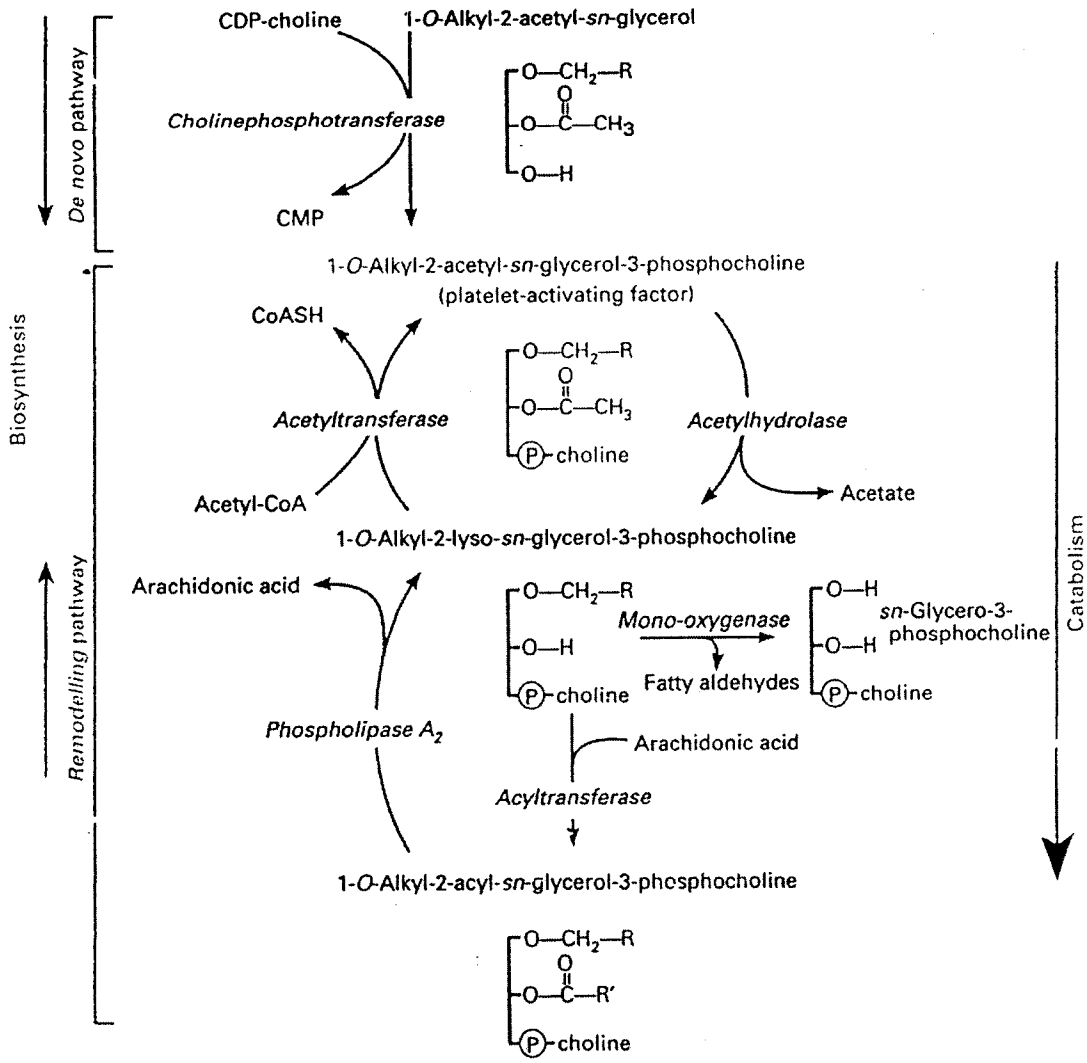


Figure1. Metabolic pathways of PAF (Chao and Olson, 1993).

PAF is produced by a wide variety of human cells and tissues, including granulocytes, macrophages, endothelial cells, lung, brain and kidney (Peplow, 1999). Activation of cellular responses is mediated by the interaction of PAF with its specific receptor. PAF binding sites were identified in numerous cells and tissues, including

human platelets, neutrophils, eosinophils, leukocytes, macrophages, human lung, liver, retina, uterus and brain (Hwang, 1990; Izumi and Shimizu, 1995).

The PAF receptor (PAFR) was originally cloned from guinea pig lung, employing the techniques of in vitro transcription and functional expression in *Xenopus laevis* oocytes (Honda et al., 1991). The human PAF receptor was isolated from leukocytes by a homology-probing approach (Nakamura et al., 1991; Kunz et al., 1992).

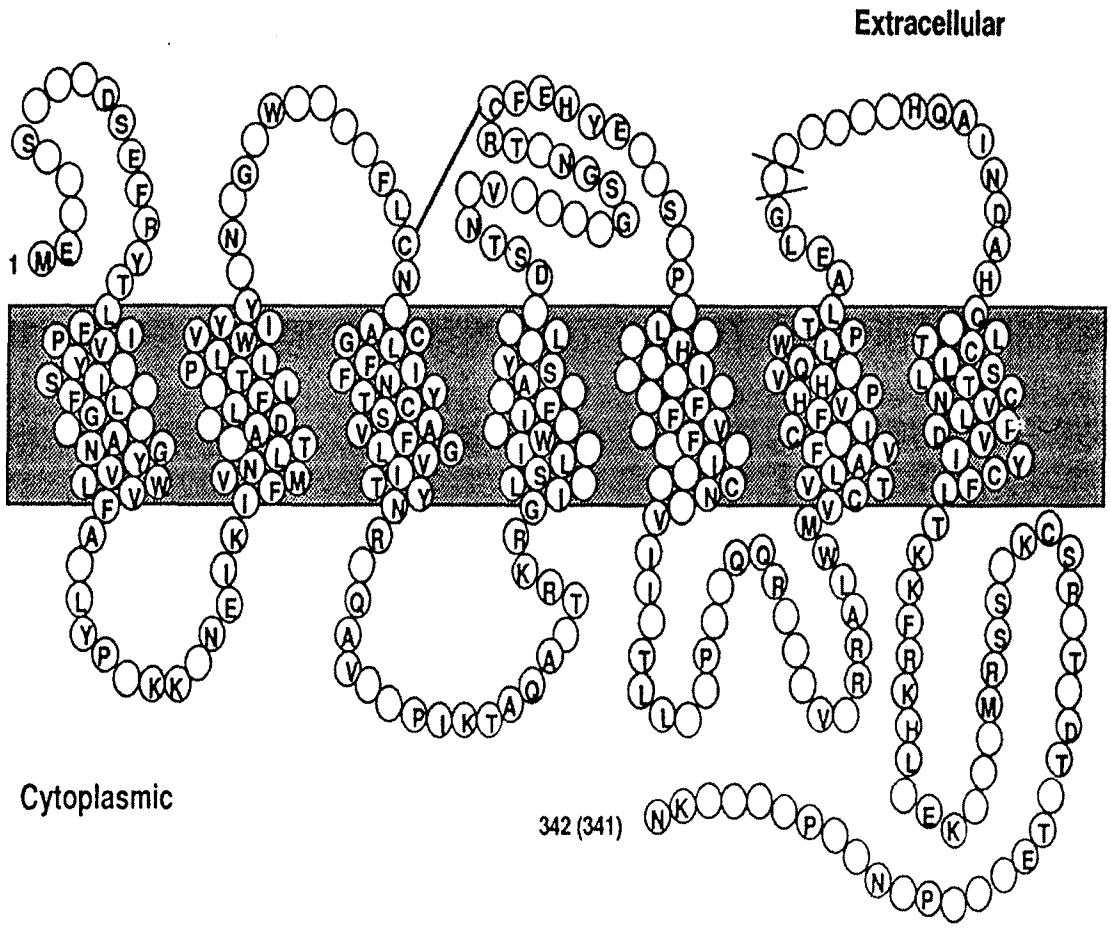


Figure 2. Model of cloned PAF receptors. Identical amino acids between 4 species are shown as single letters (Shimizu and Mutoh, 1997).

Subsequently, rat and mouse PAFR homologues have been described (Bito et al., 1994; Ishii et al., 1997). The human and guinea-pig PAF receptors have 342 amino acids,

but the rat PAFR lacks one amino acid in the third extracellular loop. The overall sequence identity among the three species is 74%, with 81% identity observed in the transmembrane spanning domains (Figure 2). Analysis of the PAFR cDNA indicates that the receptor contains putative seven transmembrane segments characteristic of the G-protein coupled receptor superfamily (Izumi et al., 1995).

PAF-mediated cell activation can be inhibited by a variety of structurally different receptor antagonists (Hwang, 1990). There are three classes of PAF antagonists: natural products (isolated from herbal plants), synthetic phospholipid analogs, and other synthetic compounds (Ishii and Shimizu, 2000).

A B

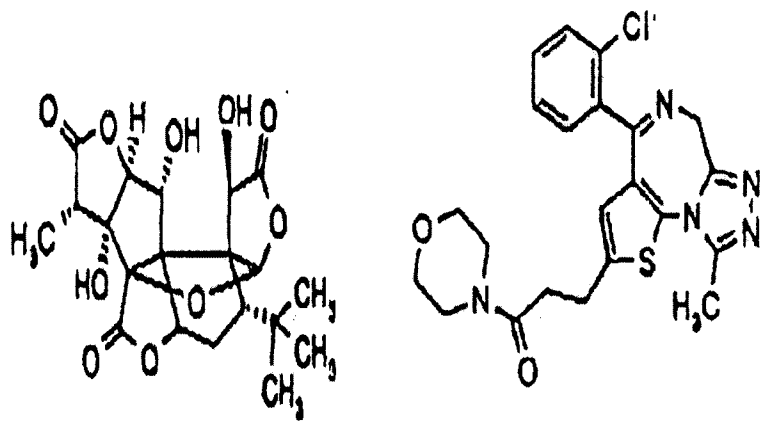


Figure 3. PAF receptor antagonists. A. Ginkgolide B. B. WEB 2086 (Curtin, 1998)

One representative natural PAF antagonist is BN 52021 or Ginkgolide B (Figure 3A). This compound is a terpenoid derived from the leaves of *Ginkgo biloba,* and is a competitive PAF antagonist (Braquet et al., 1991). Many compounds with structures similar to PAF have been synthesized including CV-3988 and CV6209 (Terashita et al., 1983). WEB 2086, which is also known as Apafant, has been used in a large number of publications (Figure 3B). This drug has been derived from an anxiolytic

triazolobenzodiazepine. Its chemical structure is not analogous to that of PAF or other lipids, but the specificity and potency to antagonize PAF-mediated effects are high (Cassals-Stenzel et al., 1987).

The human platelet-activating factor receptor gene exists as a single copy on chromosome 1 and generates two different species of mRNAs, PAFR transcript 1 and transcript 2, whose expression is driven by two distinct promoters. Two 5'-noncoding exons are alternatively spliced to the third exon, which contains an open reading frame (Figure 4, Izumi et al., 1995; Shimizu et al., 1996).

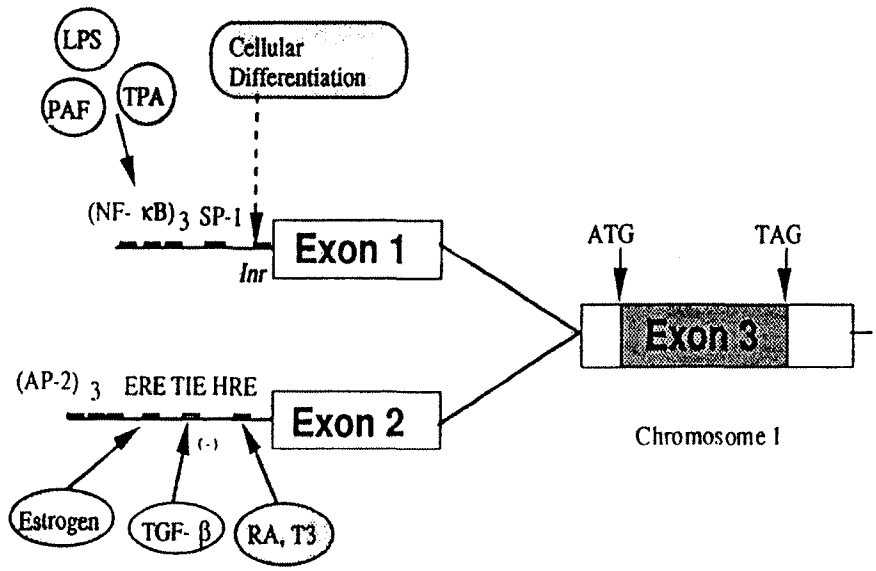


Figure 4. Genomic structure of human PAF receptor gene. ERE, estrogen responsive element; TIE, TGF-β-inhibitory element; HRE hormone responsive element (Shimizu and Mutoh, 1997).

The human transcript 1 is ubiquitous, and most abundant in peripheral leukocytes, eosinophils and monocytes, while transcript 2 is located in the heart, lung, spleen, kidney,

skin, bronchial epithelial cells and colonic crypts, but is scarce in the brain and peripheral leukocytes (Shimizu et al., 1996; Ishii and Shimizu, 2000). In guinea-pig tissues, the expression of PAFR mRNA is most abundant in leukocytes followed by lung, spleen and kidney (Honda et al., 1991). In rat tissues, the expression of PAFR was detectable in the spleen, kidneys, lungs, liver and brain, while it was less abundant in thymus, testis, heart and skeletal muscle (Bito et al., 1992, Bito et al., 1994). We, and others, have demonstrated that PAFR gene expression can be modulated by IFNγ, transforming growth factor-β (TGF-β), cyclic adenosine monophosphate (cAMP), phorbol 12-myristate 13-acetate (PMA), and tumor necrosis factor α (TNF-α) at a transcriptional level (Ouellet et al., 1994; Dagenais et al., 1997; Thivierge et al., 1993; Yang et al., 1997; Parent and Stankova, 1993). The human PAFR transcript 1 is also regulated by lypopolysacharide (LPS) and PAF in human monocytes and alveolar macrophages (Mutoh et al., 1993; Shimizu et al., 1996; Shirasaki et al., 1994). Pang and colleagues showed that the sequence spanning nucleotides –44 to +27 of the PAFR gene can drive the transcription of the receptor in myeloid cells, thus representing the minimal promoter (Pang et al., 1995). Three repeat of NF-κB-binding site, located between –898 and –785 of the transcription initiation site of transcript (Mutoh et al., 1994), was found to be important for PAF-induced up-regulation of its receptor in a human cancer cell line (Mutoh et al., 1994). NF-κB was also critical for up-regulation of PAFR gene expression in response to TNF-α in MonoMac-1 cells (Dagenais et al., 1997a) and to mechanical stretch in pulmonary artery smooth muscle cells (Chaquour et al., 1999). The human PAFR transcript 2 is regulated by retinoic acid (RA), thyroid hormone (T_3), estrogen, and transforming growth factor β (TGF-β) (Mutoh et al., 1994a; Mutoh et al., 1996). The

levels of PAF receptor mRNAs are increased by estrogen, RA and thyroid hormone treatment in Jr-St stomach cancer cell line, human endometrial grandular cells as well as in the heart and skin of RA and T_3-deficient rats (Mutoh et al., 1994a; Mutoh et al., 1996). Three direct repeats of TGACCT-like hexamer motifs with 2 and 4 bp spacers between –66 and –45 bp (termed Box A, B, and C) in human PAFR promoter 2 are the hormone responsible element (Mutoh et al., 1996). Box A + B motifs act as RA-responsible element and Box B + C motifs act as T_3-responsible element (Mutoh et al., 1996). In contrast, TGF-β is a negative regulator of PAFR transcript 2. A negative response element for TGF-β was mapped to the sequence from –90 bp to –81 bp by chloramphenicol acetyltransferase (CAT) assay using various deletion constructs (Mutoh et al., 1994a). It seems likely that the major control mechanism of expression of the PAF receptor is at the transcriptional level. However, our group observed that the PAFR mRNA levels in human monocytes decreased in response to PKC and that the mRNA half-life was markedly diminished (Thivierge et al., 1996), indicating that the stability of the mRNA may represent another potential regulatory mechanism.

The majority of signal transduction processes are initiated as a result of ligand receptor interactions. The structure-function relationship of the PAFR has been studied by site-directed mutagenesis. To identify the PAF-binding site, Ishii and co-workers (Ishii et al., 1997) systematically mutated all the polar amino acids in the transmembrane domains (TM) of the guinea pig PAF receptor. They found that mutations in TM II (N58A, D63A), TM III (N100A, T101A, S104A) and TM VII (D289A) resulted in higher affinities for PAF than the wild-type (WT) receptor. Conversely, mutants in TM V (H188A), TM VI (H284A, H294A, Q252A) and TM VII (Q276A, T278A) had lower

affinities (Ishii et al., 1997). An N100A mutant in the third TM domain was constitutively active and had lost some substrate specificity. This mutant receptor, when expressed in Chinese hamster ovary (CHO) cells, responded well to lyso-PAF, an inactive derivative of PAF, which gives little or no response with the WT receptor. Our group examined the transmembrane domains and found two adjacent phenylalanines (F97 and F98) to be important for structural stability of the receptor (Parent et al., 1996). In addition, two residues N285 and D289 could be determinant in receptor conformation and activation, since mutations of N285 to isoleucine and D289 to alanine resulted in loss of ligand binding and abolished PAF-dependent inositol phosphate (IP) accumulation, respectively (Parent et al., 1996). Recently, our group reported that the D289A mutant failed to mediate arrestin binding and translocation, indicating the importance of this residue in both G-protein coupling and arrestin recruitment (Chen et al., 2002). We also explored the role of several cysteine residues and showed that there is a disulfide bond between the conserved residues at positions 90 and 163 (Le Gouill et al., 1997). In addition to constitutively active PAFR mutant N100A, Parent and colleagues showed that mutation of leucine 231 to arginine (L231R) resulted in increased constitutive activity of the receptor and an increased affinity for PAF (Parent et al., 1996). Interestingly, the substitution of an adjacent alanine 230 for glutamic acid (A230E) led to a marked decrease in activity and affinity for agonist. Another series of studies defined the regions of the receptor important for PAF-induced intracellular signals. The third intracellular loop has been implicated by studies from multiple laboratories. Carlson and co-workers generated minigene constructs encoding different intracellular loops of the rat PAFR. They showed that a minigene encoding the third intracellular loop of the receptor inhibits

PAF-stimulated IP production. They also demonstrated that chimeric receptors that included or lacked this region could shift the signaling response (Carlson et al., 1996). Later, the same group reported the identification of a putative amphipatic helix in the aminoterminal region of the third intracellular loop of the rat PAFR (Carlson et al., 1998). Substitutions introduced into hydrophobic or polar faces of this helix completely prevented coupling of rat PAFR to G proteins and PAF-stimulated signaling without altering receptor expression (Carlson et al., 1998). From these experiments, it seems clear that the third intracellular loop of the receptor is critically important for G-protein coupling and initiating phosphatidylinositol turnover. Our group reported that aspartate 63 residue in TM domain II of human PAFR may also be involved in the structural requirements for G-protein coupling. The mutant D63N receptor showed higher affinity for PAF when compared to the WT receptor but failed to induce inositol phosphate production following agonist stimulation (Parent et al., 1996). Recently, Fukunaga and collaborators identified a naturally occurring mutation in the human PAFR gene in the Japanese population. Functionally, the mutation of an aspartic acid at position 224 to an alanine residue resulted in a significant reduction of PAF-induced signaling, such as IP production, Ca^{2+} mobilization, inhibition of adenylate cyclase, and chemotaxis (Fukunaga et al., 2001).

Rodriguez and colleagues (1995) studied the role of N-glycosylation in PAF receptor function. There is a single N-linked glycosylation site, N169, located in the second extracellular domain (Kunz et al., 1992). The mutant N169A receptor has the same affinity for PAF as the WT receptor, but its cell surface expression was greatly reduced, consistent with a requirement for glycosylation for efficient cell surface

transport (Rodriguez et al., 1995). As other GPCRs, the PAF receptor is desensitized after its initial activation. Ali and co-workers found that the desensitization of the human PAFR was accompanied by phosphorylation, which was blocked by protein kinase C (PKC) inhibitors (Ali et al., 1994). However, another group found that a PAF receptor that lacked the C-terminal intracellular region and another mutant, in which several serine residues and one threonine residue were replaced with alanine, could be desensitized and pretreatment with PKC inhibitors had no effect (Takano et al., 1994). This group also showed that a peptide representing the carboxyl-terminal 18 amino acids of the receptor was an excellent substrate for the G-protein-coupled receptor kinase-2 (GRK2) (Takano et al., 1994). Taken together, these experiments showed there are different mechanisms of the PAFR desensitization. The C-tail of the PAFR may be the target for phosphorylation which may be required to desensitize the receptor. Recently, our group demonstrated that PAF-induced internalization of the receptor depends on arrestins and the C-terminal residues from 318 to 330 are required for their association with the receptor and ligand-mediated translocation (Chen et al., 2002).

PAFR transgenic models

Three different animal models have been created to study the role of PAFR in vivo. In the first, Ishii and colleagues overexpressed the guinea pig PAFR in mice (Ishii et al., 1997). The transgenic animals had increased mortality, when challenged with endotoxin, developed melanocytic tumors of the skin, and had increased bronchial hyperreactivity in response to inhaled methacholine. The same group also created mice with a targeted deletion of the PAF receptor (Ishii et al., 1998). These PAFR knockout animals were developmentally normal and reproduced effectively, indicating that PAF is

unlikely to be essential for murine reproduction. However, aberrant expression of PAF receptor appears to prevent normal ontogeny by collapsing the regulated PAFR expression in embryo and uterus (Ishii et al., 1998; Ishii and Shimizu, 2000). PAFR knockout animals were tested in a model of inflammation and had much milder anaphylactic responses to exogenous antigen challenge than did WT animals, including less cardiovascular instability, airway contraction, and alveolar edema (Ishii et al., 1999). In the third study, Shimizu and colleagues examined inflammatory acute lung injury, induced by acid aspiration in knockout mice and in transgenic mice overexpressing the PAFR. The injury was reduced in animal deficient in the PAF receptor and increased in the overexpressing mice (Nagase et al., 1999).

PAFR-mediated signaling

PAF-receptor-induced signals involve guanine nucleotide regulatory proteins (G proteins). G proteins play important roles in determining the specificity and temporal characteristics of the cellular responses to signals. They are made up of α, β, and γ subunits (Hamm, 1998). Classification of G proteins is based on the nature of the Gα subunit. Four main classes of G proteins can be distinguished: G_s (including G_s, G_{XLS} and G_{olf}), G_i (G_{i1}, G_{i2}, G_{i2L}, G_{i3}, G_{o1}, G_{o2}, G_{gust}, $G_{tr,c}$, G_z), G_q (G_q, G_{11}, G_{14}, G_{15}, G_{16}) and G_{12} (G_{12}, G_{13}) (Nurnberg and Ahnert-Hilber, 1996). Six β and 13 γ gene products are known, giving rise the diversity of G proteins by the formation of different combinations with Gα subunits (Clapham, 1997). In the G-protein family, G_s is known to activate adenylate cyclase, G_i inhibits this enzyme and G_q induces activation of phospholipase C (PLC)

(Figure 5; Hamm, 1998). G_{12} subfamily members are known to modulate Na^+/H^+ exchange (Fields and Casey, 1997).

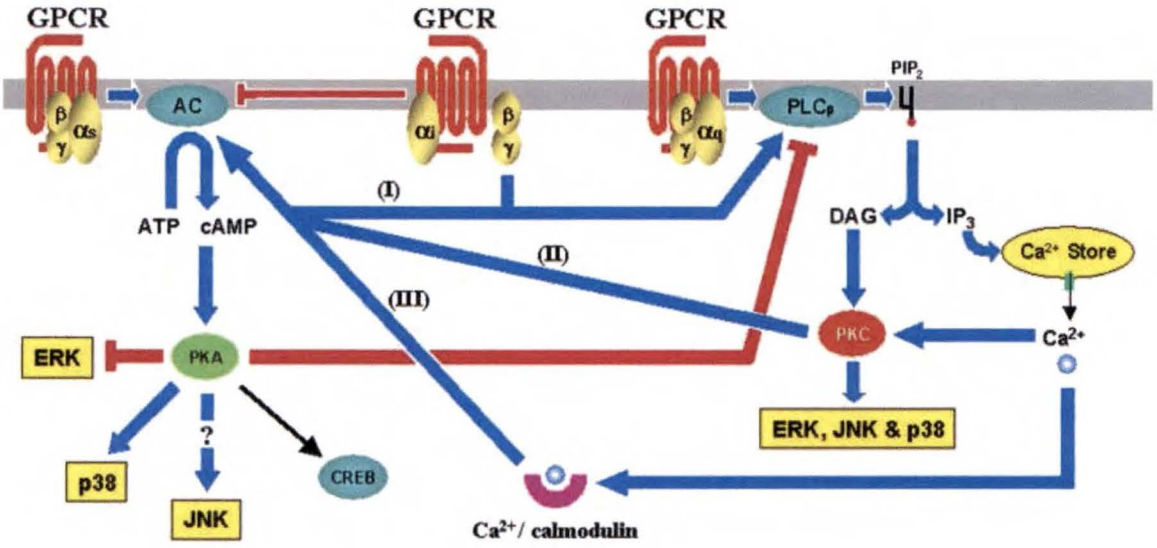


Figure 5. G-protein-dependent signaling cascade.

In the classical paradigm of GPCR activation, the first step is ligand-induced conformational changes of the receptor. These changes then affect the conformation of the G-protein-interacting intracellular loops of the receptor and thus uncover previously masked G-protein binding sites. G proteins are inactive in the GDP-bound, heterotrimeric state and are activated by receptor-catalyzed guanidine nucleotide exchange, resulting in GTP binding to the α subunit. GTP binding leads to dissociation of Gα-GTP from Gβγ subunits and activation of downstream effectors by both Gα-GTP and free Gβγ. The interaction with effectors then stimulates the intrinsic GTPase activity of Gα subunit that hydrolyzes GTP to GDP. Gα-GDP reassociates with Gβγ thus terminating the activation

cycle (Nurnberg and Ahnert-Hilber, 1996, Hamm, 1998). One of the key events in PAF-mediated signaling mechanisms through G-proteins is the hydrolysis of phosphatidylinositol 4,5-biphosphate, by a specific phospholipase C, yielding two second messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate. DAG then activates protein kinase C leading to phosphorylation of various substrates whereas inositol 1,4,5-triphosphate mobilizes intracellular calcium (Chao and Olson, 1993).

Several molecules and toxins have been used to investigate the activation and signalisation through G proteins. Among them, analogs of GTP (GTPγS and GppNHp), aluminum fluoride (AlF_4^-), and cholera toxin represent excellent activators of G proteins. Several studies have used pertussis toxin (PTX) as a tool to monitor the involvement of G_i proteins in PLC activation. PTX induces ADP-ribosylation of the cysteine residue of $G\alpha_i$ subunits and prevents their interaction with a receptor (Bras et al., 1988). A well-established pathway for activation of PLCβ is through the subunits of PTX-insensitive G_q, G_{11}, G_{14} and G_{16} proteins. The βγ subunits can also activate PLCβ and this is thought to be the mechanism for PTX-sensitive activation of PLC (Izumi and Shimizu, 1995, Ishii and Shimizu, 2000). PAF activates phosphoinositide turnover via PLC in many systems, including platelets, monocytes, neutrophils, a human B cell line, smooth muscle cells, among others (Izumi and Shimizu, 1995; Chao and Olson, 1993; Shukla, 1991). In macrophages and neutrophils the phosphoinositide turnover induced by PAF is sensitive to PTX (Hung et al., 1988; Naccache et al., 1985), while in platelets and U937 cells it is insensitive to PTX (Barzaghi et al., 1989; Dhar et al., 1990). When the cloned PAF receptor was expressed in CHO cells, PAF-stimulated inositol phosphate (IP) production was insensitive to PTX (Honda et al., 1994), while when it was expressed in RBL-2H3

cells it was partially sensitive (Ali et al., 1994). In HN33.11 cells (immortalized hippocampal cell line), antiserum against $G\alpha_{q/11}$ attenuated the IP formation by 70%, while PTX and anti-$G\alpha_{i1/2}$ antiserum also showed attenuation by 20-30% (Shi et al., 1996). PAF also activates PLCγ in a human B cell line, with the use of inhibitors, Kuruvilla and colleagues demonstrated that protein tyrosine kinases might be upstream of PLCγ and induce its activation (Kuruvilla et al., 1994). PAF caused the association and phosphorylation of both c-Src and PLCγ1 in rabbit platelets, suggesting direct phosphorylation of PLCγ1 by c-Src (Dhar and Shukla, 1994). In RBL-2H3 rat basophilic cells expressing PAF receptor, PAF activates $PLC\beta_3$ (Ali et al., 1997). These data indicate that PAF activates PLC via coupling to both PTX-sensitive and PTX-insensitive G proteins. The type of G proteins involved in PAF responses could vary according to cell type and effector molecules expressed in a particular cellular system.

PAF also stimulates gene expression including the expression of primary response genes such as Cot2 in A-431 cells (Tripathi et al., 1991), c-fos in human monocytes (Ho et al., 1987), B cell lines (Mazer et al., 1991) and epithelial cells (Bazan et al., 1993), and c-jun in human neuroblastoma and epithelial cells (Bazan et al., 1993). These primary response genes appear to play important roles in cell growth and differentiation. PAF also up-regulates cytokine and cytokine receptor genes such as IL-1, TNF, IL-2Rα in human monocytes, IL-6 in human neutrophils, monocytes, smooth muscle cells, endothelial and epithelial cells (Rola-Pleszczynzki et al., 1993).

PAF also activates PLA_2, PLD and mitogen-activated kinase (MAPK) in many different cells and tissues (Izumi and Shimizu, 1995; Liu et al., 1994). It induces tyrosine

phosphorylation of numerous cellular proteins, such as $p125^{fak}$ in human endothelial cells and brain (Soldi et al., 1996; Calcerrada et al., 1999), p85 regulatory subunit of phosphatidyl inositol 3-kinase (PI3K) (Kuruvilla et al., 1994), $pp60^{src}$ (Dhar et al., 1994), and Fyn, Syk and Lyn in a human B cell line (Kuruvilla et al., 1994, Rezaul et al., 1997). Rapid activation of small G-proteins, Ras, Rap1, Ral, p21rac, was observed in human neutrophils stimulated with PAF (Coffer et al., 1998: M'Rabet et al., 1998: M'Rabet et al., 1999; Geijen et al., 1999). Ral activation was PTX-insensitive, and was mediated by Src-like tyrosine kinases and PI3K. PAF-induced Ras activation in human neutrophils is also mediated by Src-like tyrosine kinases, but is independent of PI3K (M'Rabet et al., 1999). The rapid activation of Ras was also reported in human eosinophils (Coffer et al., 1998a).

There are three different classes of highly conserved MAPK: extracellular signal-regulated kinases (ERKs), p38, and c-Jun N-terminal/stress-activated protein kinases (JNKs/SAPKs). In primary hippocampal neurons, PAF activation of ERK, JNK, and p38 was reported (De Coster et al., 1998) However, multiple pathways seem to exist for the activation of MAPK by the PAF receptor, depending on species and cell type (Ishii and Shimizu, 2000). PAF-triggered activation of ERK was first reported in a human B lymphoblastoid cell line LA350 (Frankli et al., 1993). Later, Lefkowitz's group reported MAPK activation in CHO cells expressing the cloned PAFR. They showed that ERK activation was mediated by Go subunits (van Biesen et al., 1996). This signaling pathway required activity of PKC but was independent of Ras activation. PAF-stimulated p38 activation in CHO cells was PTX-insensitive and attenuated by the GTPase-activating protein RGS16 (Zhang et al., 1999). In PAF-stimulated human neutrophils, the activation

of p38 and ERK1 and ERK2 was also reported, the activation of p38 was PTX-insensitive and much stronger than that of ERKs (Nick et al., 1997). With the use of inhibitors, another group showed that MEK1/MEK2 and PI3K might be upstream of ERKs in PAF-stimulated human neutrophils (Coffer et al., 1998).

Several mechanisms are now known to mediate MAPK activation by GPCR. One of them is GPCR-induced transactivation of the Epidermal Growth Factor Receptor (EGFR). Activation of a GPCR leads to the release of a metalloprotease that in turn leads to release heparin binding EGF (HB-EGF). This HB-EGF acts in an autocrine/paracrine manner to activate EGFR. Phosphorylated EGFR recruits other signaling molecules, such as Shc-Grb2-Sos1. The recruitment of Grb2-Sos leads to ERK activation via sequential activation of Ras, Raf and MEK (Pierce et al., 2001). Implication of this mechanism in ERK activation was elegantly demonstrated by Prenzel and co-workers (Prenzel et al., 1999) for several GPCRs including thrombin receptor, LPA receptor, bombesin and endothelin receptors. Another mechanism was proposed from studies of ERK activation by β_2 adrenergic (β_2 AR) and neurokinin-1 (NK-1) receptors (DeFea et al., 2000; Luttrell et al., 1999). In this case, agonist binding results in receptor phosphorylation by GRK and recruitment of c-Src-β-arrestin complex that leads to ERK activation. Recently, McDonald and colleagues discovered that β-arrestin-2 could interact directly with the MAPKKK and MAPK (McDonald et al., 2000). Our group recently found that the dominant negative Jak2 or Tyk2 inhibited PAF-stimulated ERK activation in HEK 293 cells transiently expressing PAFR, suggesting that Jak/STAT pathway is upstream of the Ras/Raf/MEK/ERK pathway (Chen et al., manuscript in preparation).

Jak/STAT pathway

The first Jak kinase, named Tyk2, was obtained upon screening a T-cell library using low stringency hybridization techniques (Krolewski et al., 1990: Firmbach-Kraft et al., 1990). The unique structure and function of Tyk2 became obvious only after other members of the Jak family were characterized. Partial cDNA clones of Jak1 and Jak2 were obtained by PCR using oligonucleotide spanning the conserved kinase domains of members of the Src family of protein tyrosine kinases (Wilks, 1989). Full-length cDNA clones of Jak1 and Jak2 were subsequently isolated using the partial cDNA fragments as probes (Wilks, 1991; Harpur, 1992). Several independent groups discovered the fourth mammalian member of the Jak family, Jak3 (Johnston et al., 1994; Rane et al., 1994; Kawamura, 1994; Takahashi and Shirasawa, 1994; Witthuhn, 1994). Jak1, Jak2 and Tyk2 are ubiquitously expressed and Jak3 is expressed predominantly in cells of hematopoietic origin, although low Jak3 expression was reported, recently, in normal and transformed human cell types of various origins (Lai et al., 1995; Verbsky et al., 1996). Jaks are also found in *Xenopus laevis*, *Caenorhabtidis elegans* and fish.

The Janus kinase/Signal Transducer and Activator of Transcription (Jak/STAT) pathway is recognized as one of the major mechanisms by which cytokine receptors transduce intracellular signals (Figure 6). The basic paradigm is that receptor-associated Jaks rapidly become tyrosine phosphorylated and activated following ligand binding to a particular receptor. Jak activation is followed by tyrosine phosphorylation of receptor subunits, providing binding sites for several SH2-domain-containing proteins, including STATs, which will then bind to the phosphorylated receptor and themselves become targets for Jaks. Phosphorylation of STATs results in their homo- or hetero-dimerization

via reciprocal phosphotyrosine-SH2 domain interaction. These transcription factors then translocate to the nucleus using the importin α/β localization pathway and activate downstream gene transcription (Rane and Reddy, 2000; Liu et al., 1998).

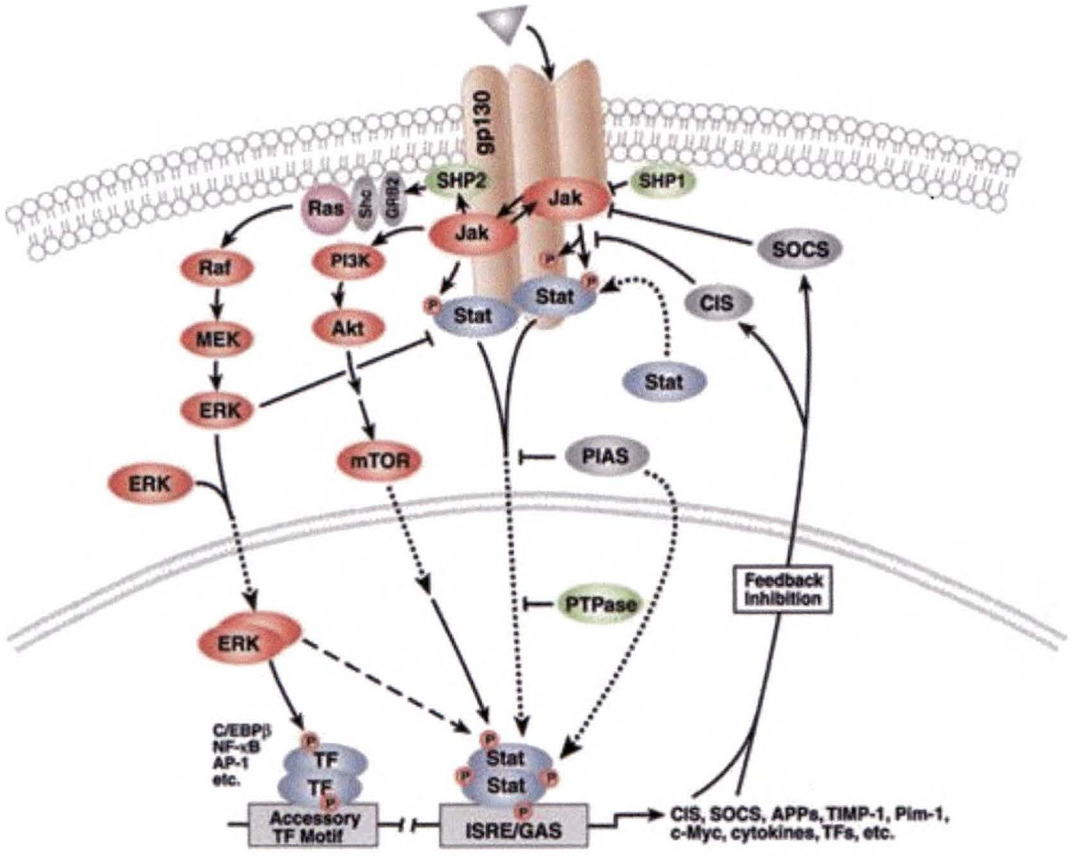


Figure 6. Jak/STAT signaling cascade: IL-6 receptor family.

Recent crystallographic and fluorescent data from studies of the erythropoietin receptor (EPOR) complex showed that, the nonligated EPOR complex exist as a preformed dimer. The intracellular domains, which are associated with the Jak2 molecules, are approximately 73 Å apart. Binding of EPO to the extracellular domain of the receptor induces a conformational change that brings receptor intracellular chains

within 39 Å of each other, allowing the activation of Jak2 (Livnah et al., 1999; Remy et al., 1999).

The dominant negative mutation of the *Drosophila* Jak homolog, Hop, results in hematopoietic defects (Binari and Perrimon, 1994). No function of the Jak kinases in mammalian cells was known until their function in interferon (IFN) signaling was recognized. The essential role of Jak family kinases in IFNα/β as well as IFNγ signaling was elegantly demonstrated by several independent groups. A number of cell lines, mutant in different proteins in the IFNα and IFNγ signaling pathways have been developed. One of these cell lines, U1, could be restored to IFNα responsiveness by transfection of a cDNA clone encoding Tyk2 (Velazquez et al., 1992). The γ1 mutant cell line, which failed to respond to IFNγ but retained IFNα/β responsiveness, lacks Jak2. The U4 mutant cell line barely responded to either IFNα/β or IFNγ. Complementing with Jak1 restored IFN-induced gene expression. Taken together, these studies showed that Jak1 and Tyk2 were essential for IFNα/β signaling and that Jak1 and Jak2 were required for IFNγ signaling (Watling et al., 1993; Muller et al., 1993; Shindler and Darnell, 1995). Later, Ihle's group examined the role of Jak kinases in EPO receptor signaling and found that Jak2 is activated in response to EPO (Willhuhn et al., 1993). Subsequent studies have revealed that dozens of soluble mediators ranging from growth factors to hormonal polypeptides and interleukins utilized Jaks as intracellular effectors (Pellegrini and Dusanter-Fourt, 1997; Watanabe and Arai, 1996; Duhe et al., 2001).

The Jak proteins are highly related protein tyrosine kinases of 120-140 kDa, characterized by an amino-terminal portion of approximately 600 amino acids and two kinase domains. The structural domains featured in the Jak kinase family are referred to

as the Jak homology regions (JH1-JH7). The tyrosine kinase domain (JH1) at the C-terminus of the protein is flanked by a central kinase-like domain (KL or JH2) unique to the family. Five JH regions of variable length and degrees of identity (JH7-JH3) follow a non-conserved amino terminus of about 30-50 amino acids (Figure 7, Pellegrini and Dusanter-Fourt, 1997).

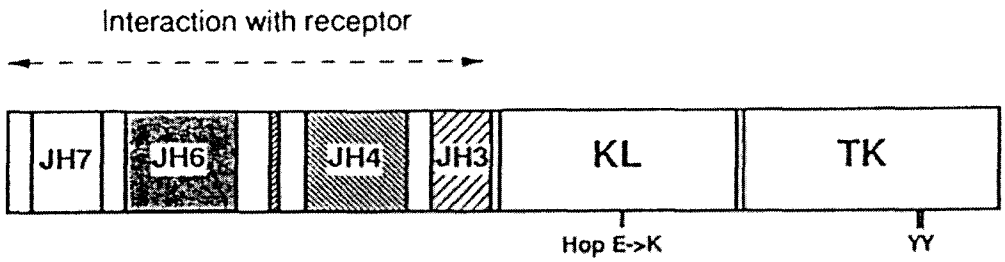


Figure 7. Structural organization of Jaks. TK, tyrosine kinase domain; KL, kinase-like domain. YY are two adjacent and conserved tyrosine residues in the activation loop of the tyrosine kinase domain. The location of the E695K mutation in the hyperactive *Drosophila* Hop mutant is indicated (Pellegrini and Dusanter-Fourt, 1997).

Recent studies suggest the existence of two binding domains that mediate cytokine receptor binding by a given Jak. One domain would be in the JH6-7 region, and a second would be in the JH3-5 region. In the case of Jak2, binding to the growth hormone (GH) receptor appears to require the entire amino-terminal half of the protein (JH3-JH7) (Tanner et al., 1995). In contrast, granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor binds only the JH6 and JH7 domains of Jak2, although this

binding is quite weak (Zhao et al., 1995). A region encompassing the JH6 and JH7 domains of Jak3 is sufficient for the interaction of the kinase with the common γ-chain of the interleukin-2 (IL-2) receptor. Krolewski's group observed that binding of IFNaR1 to Tyk2 requires amino acids 1-601 of Tyk2 containing the JH3-7 domains, and within this region, the JH3 and JH6 domains showed some independent binding (Yan et al., 1998). Velazquez and colleagues demonstrated that both kinase domains of Tyk2 are necessary for the enzymatic activity of the protein since deletion of either domain generates a kinase-inactive protein. Moreover, both Tyk2 kinase domains are critical in order to restore a response to both IFN species (Velazquez et al., 1995). The importance of the kinase-like domain of Tyk2 for both, Tyk2 catalytic activity and the establishment of a high-affinity IFNα receptor complex, was further highlighted by Yeh and colleagues (Yeh et al., 2000). This group identified four point mutations in the kinase-like domain of Tyk2 that are responsible for loss-of function phenotypes. All four Tyk2 mutants were capable of sustaining IFNaR1 expression comparably to WT Tyk2. However, two mutant proteins, H669P and R856G, were catalytically impaired and could not rescue high-affinity IFNα binding. Despite increased constitutive hyperphosphorylation, these mutant proteins failed to induce STAT1 and STAT2 activation. V584D and G596V mutants were 100-fold less effective in inducing gene expression and less phosphorylated in response to IFNα. The authors propose a model of Tyk2 regulation in the IFNα receptor-kinase complex (Figure 8). The base of this model is that the kinase-like (JH2) domain maintains the kinase domain in an unphosphorylated and resting conformation. Upon IFNα binding, JH2-dependent conformational changes allow transition of the receptor-

kinase complex to a high-affinity binding state where Tyk2 and Jak1 are stabilized in their activated conformation (Yeh et al., 2000).

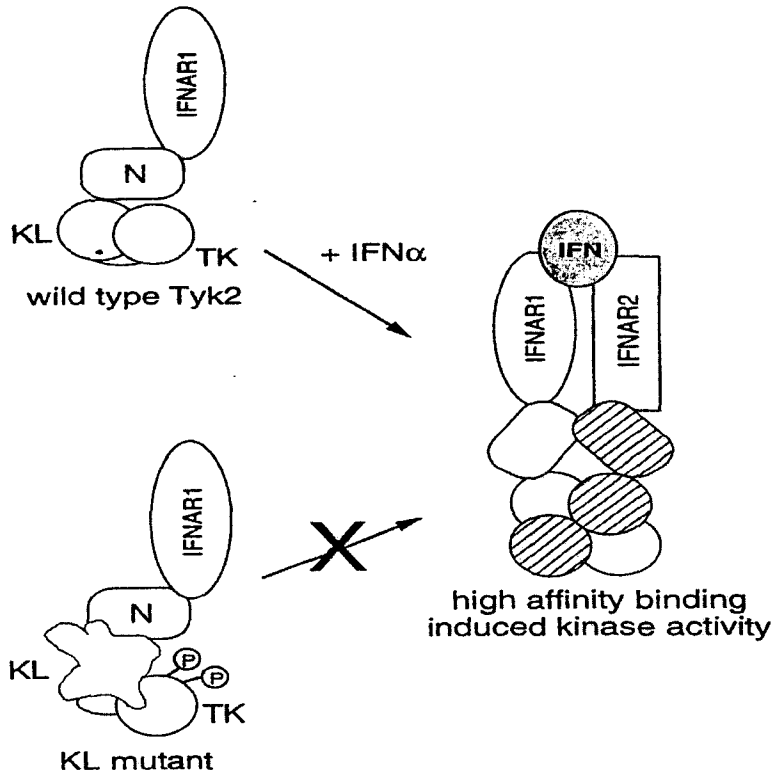


Figure 8. A model of Tyk2 regulation in the IFN-β receptor-kinase complex (Yeh et al., 2000).

Saharinen and colleagues (2000) recently ascribed a regulatory role to the Jak2 pseudo-kinase domain in the modulation of Jak2 kinase activity. The authors demonstrated that deletion of the Jak2 pseudo-kinase domain negatively regulated Jak2 catalytic activity as well as STAT5 activation by Jak2. Moreover, Jak2 kinase inhibition was mediated by an interaction between the kinase and kinase-like domains (Saharinen et al., 2000).

Membrane proximal domains of cytokine receptors were found to mediate Jak binding (Pellegrini and Dusanter-Fourt, 1997). The Box 1 motif comprising approximately eight proline-rich amino acids is required for Jak2 interaction with several cytokine receptors, for example, EPOR (Witthuhn et al., 1993; Miura et al., 1994), GHR (VanderKuur et al., 1994; Hackett et al., 1995), prolactin receptor (DaSilva, et al., 1994), and gp130 (Tanner et al., 1995). Box-1-like motifs were also found in IFN receptors. In the IFNγR1 subunit, the site for Jak1 association is in the membrane-proximal region and contains an essential proline residue in a Box-1-like motif, LPKS (Kaplan et al., 1996). The IFNRγ2 subunit contains two juxtaposed sequences important for signaling, one of which is rich in proline residues (PPSIP) and critical for formation of a Jak2-binding site (Bach et al., 1996). On the other hand, the Tyk2 binding site on the IFNaR1 receptor represents a membrane-proximal 33 amino acid sequence, with no essential proline residues (Yan et al., 1996). The requirement of membrane proximal motifs in Jak kinase interaction is absolute even in the case of receptors, lacking Box 1 motifs. The IL-2R γc chain does not contain a typical Box 1 motif, but a membrane proximal region encompassing 52 residues is sufficient for Jak3 binding, activation of Jak1 and Jak3 and other downstream signaling molecules (Nelson et al., 1996).

Presently there are two hypotheses how the oligomerization of the receptor-associated Jaks results in a change of catalytic activity of the enzyme. One explanation is that the enzyme exists in a low activity state prior to receptor stimulation, and is then further activated via trans-phosphorylation. Upon receptor stimulation, the catalytic domain of one Jak transphosphorylates a tyrosine within the activation loop of the neighboring Jak (Duhe et al., 2001). Several studies support this idea. Substitution of

Tyr1054 in Tyk2 (Gauzzi et al., 1996), Tyr1007 in Jak2 (Feng et al., 1997) and Tyr980 in Jak3 (Zhou et al., 1997) to phenylalanine residue results in enzymes unresponsive to receptor stimulation. Other evidence confirming trans-phosphorylation mechanism can be found in the observation of kinase hierarchy in Jak-hetero-oligomers. A dominant-negative Jak1 prevents the activation of Tyk2 after IFN-β stimulation in 293T cells (Briscoe et al., 1996), and an inactive Jak2 blocks the activation of Jak1 in response to IFN-β receptor stimulation (Krishnan et al., 1997).

A second hypothesis of how oligomerization may lead to Jak activation is based on the presumption that the monomeric Jaks are initially in an autoinhibitory conformation, and the conformation of the oligomeric Jaks relieves this autoinhibition (Duhe et al., 2001). Although the autoinhibition proposal has yet to be proven, several reports also support this idea. Truncation of the N-terminus (JH7-JH3) of Jak2 resulted in a hyperactive protein (Duhe and Farrar, 1995). Truncation of Jak3, to produce the JH1 domain, also results in a hyperactive kinase, capable of phosphorylating STAT1 to a much higher extent than the wild-type Jak3 (Witthuhn et al., 1999).

It becomes clear that Jaks phosphorylate multiple substrates, among which are juxtaposed Jaks, receptor components and signaling proteins that are recruited to the receptor-Jak complex upon ligand binding (Rane and Reddy, 2000). GST fusion proteins encoding intracellular domains of a number of cytokine receptors could be phosphorylated by activated Jak kinases *in vitro* (Zhao et al., 1995, Colamonici et al., 1994; Gauzzi et al., 1996). The group of Kraft analyzed the pattern of tyrosine-phosphorylated proteins induced by antibody cross-linking of a CD16/Jak2 chimera in BA/F3 cells (Sakai et al., 1995). A set of proteins was found to be phosphorylated by

Jaks, among which were STAT5, Shc and the SHIP tyrosine phosphatase. A chimeric construct containing an intracellular domain of the epidermal growth factor (EGF) receptor and the Jak2 kinase domain was capable of inducing EGF-dependent STAT5 phosphorylation in 32D cells (Nakamura et al., 1996). Shc as well as ERK2 are activated and associated with Jak2 in response to GH stimulation (Vanderkuur et al., 1995; Winston and Hunter, 1995). Other substrates of the Jaks are protein tyrosine phosphatases, which modulate cytokine receptor signaling. The SH2-domain containing protein tyrosine phosphatase SHP-1 associates directly via its N-terminal SH2 domain with Jak2, as well as, with Tyk2 and Jak1, and dephosphorylates the kinases thus terminating EPO- and IFNα-induced signal transduction, respectively (Jiao et al., 1996; Yetter et al., 1995; David et al., 1995). Another SH2-domain containing phosphatase is SHP-2 that is generally recognized as a positive regulator of Jak-transduced cytokine signaling. SHP-2 associates directly with Jak2 and also serves as an adaptor protein between Jak-dependent signaling and other signal-transduction pathways induced by cytokine receptors (Duhe et al., 2000). Co-immunoprecipitation studies revealed that the interaction between Jak2 and SHP-2 depends on their N-terminal domains (Yin et al, 1997).

STATs are among the best-characterized Jak substrates. These transcription factors were originally described by Darnell and his co-workers as ligand-induced transcription factors in interferon-treated cells (Darnell et al., 1994; Darnell, 1997). Subsequent studies showed that STATs play an important role in signal transduction pathways induced by several cytokines and neurokines including the interleukins, the interferons, EPO, GH, prolactin, among others (Darnel, 1997). To-date, six mammalian

STAT genes have been identified, all of which encode proteins of 750-850 amino acids and are characterized by the presence of a DNA-binding domain followed by SH3 and SH2 domains (Darnell et al, 1994; Darnell, 1997). Additional forms for STAT1, 3, 4 and 5 can be generated via alternative splicing (Figure 8).

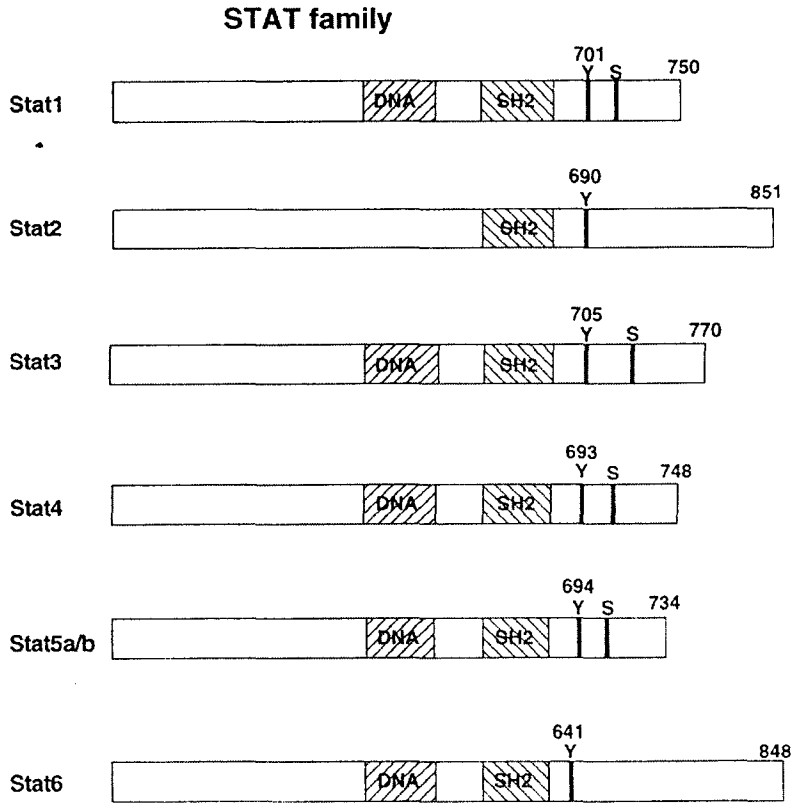


Figure 9. Structure of STAT proteins (Takeda and Akira, 2000).

The DNA binding and SH2 domains are highly conserved among the STATs. The SH2 domain is critical for the recruitment of STATs to the activated receptor complexes and is required for the interaction with Jak and Src kinases. STATs have been shown to interact directly with cytokine receptors. The interaction involves the recognition of a specific tyrosine-phosphorylated motif on the activated receptor (Pellegrini and Dusanter-Fourt, 1997). Some receptors, such as those for IL-3, IL-5, and GM-CSF, activate STATs in the absence of a tyrosine in the receptor intracellular domain (Mui et al., 1995;

Caldenhove et al., 1995). Fijitani and colleagues demonstrated an association between the kinase-like domain of Jak2 and STAT5 (Fujitani et al., 1997). Tyrosine kinase receptors recruit STATs through associated cytoplasmic tyrosine kinases. STATs might also be recruited via docking to another receptor-associated STAT, as it was demonstrated for STAT1, which requires STAT2 docking to the IFNaR1 receptor chain (Greenlund et al., 1995; Qureshi et al., 1996).

Phosphorylation of tyrosine residues immediately downstream of the SH2 domain, around position 700, is essential for the activation and dimerization of STATs. Mutation of this tyrosine residue abolishes STAT DNA-binding activity (Qureshi et al., 1996; Shuai et al., 1993; Mikita et al., 1996). Phosphorylation of this tyrosine residue could be achieved by growth hormone receptors as well as Jak and Src kinases, depending on the nature of the cell type and the ligand/receptor interaction (Darnell et al., 1994; Shindler and Darnell, 1995). Amino-terminal STAT sequences are known to mediate STAT hetero-dimerization as well as association with other, unrelated, transcription factors, for example, CBP/p300, JUN, SP1, and the glucocorticoid receptor (Pellegrini and Dusanter-Fourt, 1997; Bhattacharya et al., 1996; Look et al., 1995; Stocklin et al., 1996). The C-terminal domain of STATs is required for transcriptional transactivation. Phosphorylation of a serine residue in the transactivation domain enhances the transcriptional activity of STATs (Shindler and Darnell, 1995). Upon tyrosine phosphorylation, STATs form stable homo-or heterodimers, translocate to the nucleus and bind target DNA sequences to induce gene transcription.

The mechanisms that regulate nuclear export and import are an area of intense investigation. Accumulation of STATs in the nucleus is controlled by nuclear export

through Ran-dependent interaction with chromosome region maintenance (CRM)1/exportin 1 (McBride et al., 2000). A leucine-rich motif in the DNA-binding domain of STAT1 (residues 400-409), was found to be critical for nuclear export and represents the nuclear localization signal (NLS). Inhibition of nuclear export alone is not sufficient to cause STAT nuclear accumulation. STAT proteins are imported by the nuclear import receptor, importin-α5, and Ran. This requires STAT dimerization, and tyrosine phosphorylation alone is not sufficient (McBride et al., 2002). STAT1 L407 mutation to alanine in its NLS interfere with importin binding and nuclear import of phosphorylated STAT dimers (Melen et al., 2001; Meyer et al., 2002; McBride et al., 2002).

All STATs recognize highly related DNA sequences. Most STATs can bind to GAS elements consisting of the 9-bp palindromic sequence 5'-TTN_5AA-3'. The sequence $TTCN_3GAA$ has been defined as an optional binding site for STAT1, STAT3, STAT4 and STAT5. STAT binding to a particular DNA sequence is greatly influenced by other transcriptional factors associated with the STAT complex or bound to adjacent DNA sequences (Darnell, 1997; Pellegrini and Dusanter-Fourt, 1997).

Gene disruption studies of a number of Jaks and STATs revealed their selective roles *in vivo.* Targeted disruption of Jak2 results in an embryonic lethal phenotype highlighting the importance of this kinase for EPO, IL-3, GM-CSF and other hemotopoietic cytokines. $Jak2^{-/-}$ mice are developmentally arrested at embryonic day 13 by an inability to commence definitive hematopoiesis (Neubauer et al., 1998; Parganas et al., 1998). Jak1 is an essential mediator for biological responsiveness to a broad range of cytokines, such as the IFNs, gp130-utilizing cytokines and IL-2Rγ-chain-utilizing

cytokines. Jak1$^{-/-}$ mice fail to thrive and die perinatally within 1 day after birth (Rodig et al., 1998). The phenotype of Jak3 knockout mice closely resembles the immunological deficiencies observed in animals that lack the common cytokine receptor chain γc (Cao et al., 1995; DiSanto et al., 1995). In humans, mutations in the common γ-chain of the IL-2, IL-4, IL-7, IL-9 and IL-15 receptors or Jak3 results in severe combined immunodefficiency (SCID). Mutations found in the Jak3 gene, include a frameshift mutation, causing truncation of the JH4 domain; a nonsense mutation, leading to a deletion in the JH2 domain, a missense mutation (Y100C) in JH7 domain that prevents the protein from interacting with the IL-2 receptor (Russel et al., 1995; Macchi et al., 1995; Cacalano et al., 1999). Recently, Tyk2 knockout mice have also been generated (Kharagiosoff et al., 2000; Shimoda et al., 2000). In contrast to other Jaks, Tyk2 deficiency leads to a partial defect in the IFNα/β response and also to impaired IFNγsignaling. STAT1 and STAT2 phosphorylation was greatly reduced in Tyk2$^{-/-}$ embryonic stem (ES) cells after treatment with IFNα/β. No STAT3 and STAT2 activation was observed in Tyk2$^{-/-}$ bone marrow-derived macrophages after IFNα/β or IFNγ challenge, respectively. Moreover, Tyk2$^{-/-}$mice fail to clear vaccinia virus infection and demonstrate reduction of cytotoxic T lymphocyte activity after lymphocytic choriomeningitis virus (LCMV) challenge (Kharagiosoff et al., 2000). Another group showed that, the response to IFNα was normal in terms of STAT1 and STAT2 phosphorylation in embryonic fibroblasts from Tyk2-deficient mice, albeit at high IFNα concentrations. On the other hand, STAT4 phosphorylation, IFNγ production in Tyk2$^{-/-}$ splenocytes and differentiation of naïve helper T cells into Th1 cells were reduced in response to IL-12 (Shimoda et al., 2000). These results suggest that Tyk2 plays a

restricted role in IFNα signaling, but is critical in mediating IL-12-dependent biological responses. Taken together, studies using cellular models as well as analysis of knockout mice show that activation of Jaks is critical for such diverse responses as growth, lactation, nerve cell differentiation, hematopoiesis, and immune responses.

Jak/STAT activation by GPCRs

The Jak/STAT pathway can no longer be considered a unique feature of cytokine receptors. There is now compelling evidence of their importance in GPCR signaling. Marrero and colleagues provided the first evidence that the Jak/STAT pathway can be stimulated by the angiotensin II AT1 receptor in rat aortic smooth muscle cells (Marrero et al., 1995). Later, activation of the Jak2/STAT3 pathway was demonstrated for the CCR2B in MonoMac-1 monocytic cell line (Mellado et al., 1998) and 5-HT2a receptors in skeletal muscle myoblasts (Guillet-Deniau et al., 1997). Jak2 and Jak3 phosphorylation is induced upon ligand binding by the chemokine receptors CCR5 (Wong et al., 2001), CCR2B (Mellado et al., 1998) and CXCR4 (Vila-Coro et al., 1999). Jak2 is also stimulated by thrombin in rat vascular smooth muscle cells (Madamanchi et al., 2001) and melanocyte-stimulating hormone in mouse pro-B-lymphocyte (Ba/F3) cells, human cultured lymphocyte (IM-9) cells and mouse L-cells expressing the cloned B2 receptor (Buggy, 1998). The peptide hormone bradykinin mediated activation of the Tyk2/STAT3 pathway in bovine aortic endothelial cells (Hong et al., 2000). In most cases, Jak2 can phosphorylate the receptors (Vila-Coro et al., 1999; Mellado et al., 1998; Guillet-Deniau et al., 1997) and associates with them in a ligand-dependent manner, which is different from the cytokine receptors. Receptor regions responsible for kinase binding were shown

to be the DRY motif in the second intracellular loop of CCR2B (Mellado et al., 1998) and a proline-rich motif YIPP in the C-terminal tail of the angiotensin AT1 receptor (Ali et al., 1997). However, recent data from Marrero and co-workers indicate that binding of Jak2 to the angiotensin II AT1 receptor is indirect and occurs via the SHP-2 phosphatase which binds to the YIPP receptor motif and serves as a scaffolding protein for Jak2 (Marrero et al., 1998). Interestingly, in the case of the angiotensin AT1 receptor it appears that tyrosine residues of Jak2 but not those of receptor are important for STAT1 binding (Ali et al., 2000). Moreover, STAT1 translocation to the nucleus depends on the Jak2 motif ^{231}YRFRR, located in the amino-terminal domain of the kinase. This motif is also required for co-association of Jak2 with the AT1 receptor (Sayeski et al., 2001).

Jak/STAT regulation – phosphatases, PIASs, SOCSs

Over the years, the majority of investigations of cytokines have focused on the mechanisms by which they exert their action. It is clear, however, that the action of all of cytokines are limited in both magnitude and duration, and an understanding of the mechanisms by which their actions are negatively controlled is therefore essential. There are several different levels at which negative regulation can be achieved, including downregulation of the receptor/ligand complex (Goldstein et al., 1985), degradation of signaling intermediates, inactivation of positive regulators by dephosphorylation and activation of specific suppressors.

Early observations indicated that phosphatase inhibitors could, to some extent, mimic the action of cytokines in mitogenic responses (Tojo et al., 1987). Subsequently, the SH2-domain containing protein tyrosine phosphatase SHP-1 was identified as a

critical regulator of signal transduction pathways. The importance of SHP-1 in regulating hematopoietic development is clearly demonstrated in the moth-eaten mice (me/me), which lack expression of SHP-1 (Shultz et al., 1997, Tsui et al., 1993, Shultz et al., 1993). Moth-eaten mice display multiple hematopoietic abnormalities, including hyperproliferation and abnormal activation of granulocytes and macrophages, and an autoimmune-like phenotype. These defects can be attributed to the finding that SHP-1 suppresses signals, mediated by a variety of cytokine receptors and receptors tyrosine kinases, including receptors for EPO, IL-3, IFN-α, colony-stimulating factor 1 (CSF-1) and Steel factor (Starr et al., 1999). SHP-1 has been shown to downregulate EPO-induced proliferative signals by binding to the tyrosine phosphorylated EPOR (Y425) and dephosphorylating Jak2 (Klingmuller et al., 1995). SHP-1 has been shown to directly associate with, and dephosphorylate Jak2; this association being independent of the SH2 domain (Jiao et al., 1996). By contrast, in some circumstances SHP-1 has been noted to have a role in promoting signal transduction. For example, the expression of a catalytically inactive form of SHP-1 in HeLa cells suppressed EGF- and IFNγ-induced STAT activation (You et al., 1997). The related phosphatase SHP-2 has been demonstrated to downregulate signaling through gp130 (Symes et al., 1997). Also, You and co-workers recently documented an important role for SHP-2 in interferon-stimulated cells, negatively regulating IFN-induced growth and apoptotic pathways (You et al., 1999). Furthermore, Yin and colleagues (1997) performed a detailed molecular characterization of specific interactions between SHP-2 and Jak kinases. These authors demonstrated that SHP-2 is tyrosine phosphorylated by Jak1 and Jak2, but not Jak3, on Y304 and Y327 via a direct association. The SHP-2 and Jak kinase association requires

the N-terminal region of Jak proteins and regions encompassing residues from 232 to 272 of the phosphatase (Yin et al., 1997). Recent data of Myers and co-workers indicate that the protein-tyrosine phosphatase PTP1B recognizes Jak2 and Tyk2 as substrates and modulates signaling responses to IFNα and IFNγ (Myers et al., 2001).

Recent reports revealed a new family of proteins induced by cytokines, which inhibit cytokine action. The first member of this family was denoted as CIS (cytokine-induced SH2-containing protein). The CIS1 gene was cloned originally as an immediate early gene, which was induced by IL-2, IL-3 and EPO. CIS1 associates with tyrosine phosphorylated EPOR and IL-3-receptor β chain following stimulation with EPO and IL-3, respectively (Yoshimura et al., 1995). It is thought that CIS1 competes with STAT5 to reduce interactions between STAT5 and phosphorylated cytokine receptors (Matsumoto et al., 1997), although the interaction between the SH2 domain of CIS1 and these receptors has not yet been demonstrated.

In 1997, the negative regulator of signal transduction (SOCS-1/JAB/SSI-1) was cloned independently by three groups in very different ways: functionally by its ability to suppress macrophage differentiation of M1 cells in response to IL-6 (Starr et al., 1997); in a yeast two-hybrid screen to find proteins that interact with the kinase domain of Jak2 (Endo et al., 1997); and on the basis of antigenic similarity to STAT3 (Naka et al., 1997). Collectively, the papers demonstrate that SOCS-1/JAB/SSI-1 is a potent inhibitor of IL-6 signaling, including IL-6-induced tyrosine phosphorylation of gp130 and STAT3. Interestingly, SOCS-1 can interact not only with Jak2, but also with Jak1, Jak3 and Tyk2 and inhibit their catalytic activity (Endo et al., 1997; Naka et al., 1997). When constitutively expressed, SOCS-1 inhibits signal transduction in response to leukemia

inhibitory factor (LIF), oncostatin M (OM), thrombopoietin (TPO), IFNγ (Starr et al., 1999) and GH (Adams et al., 1998), suggesting that SOCS proteins may play central roles in determining the intensity and duration of cellular responses to many cytokines. SOCS-2/SSI-2/CIS-2 and SOCS-3/SSI-3/CIS-3 have been cloned in both murine and human systems (Starr et al., 1997; Masuhara et al., 1997). At this time, the SOCS family comprises at least 8 proteins (SOCS-1 to SOCS-7, and CIS). These proteins share a similar structure with a central SH2 domain, variable in length and amino acid sequence, N-terminus and a region of homology at the C-terminus termed the SOCS box (SC/CH box). An extensive database search has identified an additional 12 proteins that contain a C-terminal SOCS box (Hilton et al., 1998). These proteins have an SH2 domain, N-terminal to the SOCS box, but otherwise contain different motifs than the SOCS proteins. The WSB proteins contain WD-40 repeats, whereas others contain ankyrin repeats, or a SPRY domain, or a class of small GTPases (RAR and RAR-like). Zhang and colleagues (Zhang et al., 1998) showed that the SOCS box of different SOCS-box containing proteins mediates interaction with elongin B and C. This finding suggests that such an interaction may couple SOCS proteins and their substrates to the proteasome degradation pathway. Recently, Ungureanu and colleagues (2002) demonstrated specific interaction between Jak2 and SOCS-1 in response to IL-3 and IFNγ. These authors found that phosphorylation of the tyrosine residue Y1007 of Jak2 was followed by its binding to SOCS-1, Jak2 polyubiquitination and degradation (Ungureanu et al., 2002). Several reports have suggested that SOCS proteins may regulate multiple signal transduction pathways. For example, SOCS-1 was identified as a negative regulator of the cytoplasmic tyrosine kinase, Tec. Unlike the interaction with Jaks, the association of SOCS-1 and Tec

is phosphorylation independent (Ohya et al., 1997). Moreover, SOCS-7 is a binding partner for the adaptor molecule Nck and phospholipase C-γ (Matuoka et al., 1997).

The inflammatory disease present in SOCS1-/- mice proved the essential negative regulatory role of SOSC-1. These mice are viable and fertile, but die at three weeks of age because of severe lymphopenia, activated T cells, fatty degeneration and necrosis of the liver. IFN-γ and TNF produced in high levels and the acute pathological changes are attenuated by the lack of STAT1, STAT6, T and NK cells. (Naka et al., 2001). Mice expressing SOCS-1, which is lacking the SOCS box gene have augmented responsiveness to IFN-γ and slowly develop a fatal inflammatory disease (Zhang et al., 2001). SOCS-2-deficient mice exhibit gigantism (Metcalf et al., 2000) and SOCS3 deficiency was embryonically lethal due to placental defects and increased erythrocytosis (Roberts et al., 2001).

Recently, the protein inhibitor of activated STAT (PIAS) family was identified using a yeast two-hybrid screen for STAT interacting proteins. PIAS1 was identified as a specific interaction partner for STAT1, and PIAS3 was cloned on the basis of homology to PIAS1 (Chung et al., 1997). A putative zinc-binding motif is conserved in the PIAS family, but no other known motifs are present. PIAS3 is expressed constitutively, but associates with STAT3 only upon stimulation of the cells with cytokines such as IL-6, OM, CNTF. It is notable that PIAS3 was able to inhibit DNA-binding activity of STAT1/STAT3 heterodimers, although PIAS3 appears likely to bind to activated STAT3 monomers. Two other mechanisms have been suggested for inactivating STATs: proteolytic degradation and implication of phosphatases. Inhibitors of proteasome activity have been shown to stabilize the activation of STATs, but it is unclear, whether the

proteasome directly targets STATs (Kim et al., 1996) or regulates signaling intermediates upstream (Haspel et al., 1996; Yu et al., 1997). Pulse-chase labeling experiments show that the half-life of activated STATs is short but turnover of the proteins themselves is slower (Haspel et al., 1996). Specific regions of the STATs critical for mediating their inactivation have been defined: removal of 50 residues in the C-terminus of STAT3 and STAT5 results in mutants which remain phosphorylated for more than 12 h (Wang et al., 1996). Deletion of the N-terminal 60 residues in STAT1 results in a molecule that remains constitutively phosphorylated (Darnell et al., 1997). In contrast to SOCS, which are induced in response to cytokines, SHP-1 and PIAS are present constitutively in cell and may function as more acute, early response regulators (Starr et al., 1999).

The Jak kinases and the molecules that regulate their activity are central to normal development and immune function, and therefore aberrant control of Jak/STAT signaling has been implicated in a number of pathological states. Defining the mechanisms by which cells regulate Jak kinase activity is of paramount importance in understanding how and why regulation fails and in understanding any resulting pathology (Losman, 1999).

II. Hypothesis and Objectives of the research project.

In the present thesis we examine the role of the Jak/STAT pathway in PAFR-mediated signaling. Our initial work was based on the observation of Marrero and colleagues (Marrero et al., 1995) who demonstrated, for the first time, activation of Jak/STAT pathway by the member of GPCR family, angiotensin II receptor AT1. In addition, we and others have also shown that PAF-stimulated PAFR gene expression is modulated by IFN-γ, cAMP, TGF-β, PMA (Ouellet et al., 1994; Dagenais et al., 1997; Thivierge et al., 1993; Yang et al., 1997; Parent and Stankova, 1993). However, the precise mechanisms, as well as the nature of the transcription factors, involved in PAF-stimulated PAFR gene expression have not been elucidated. In our study (Chapter III), we investigated the involvement of the Jak/STAT pathway in the regulation of PAFR and considered possible mechanisms of Jak kinase activation using mutant forms of PAFR, D63N, D289A and Y293A, that do not couple to G-proteins.

Since our results (Chapter III) indicate that Tyk2 associates with PAFR and is essential for PAF-induced PAFR promoter activation, we were therefore interested to determine the regions within the receptor important for kinase binding as well as for signaling (Chapter IV). Using PAFR GST-fusion proteins, bearing receptor intracellular loops and PAFR deletion and substitution mutants, we performed in vitro binding and co-immunoprecipitation studies. In addition, we characterized the mutant receptors: their cell surface expression, affinity to ligands and PAF-induced PAFR transcription.

Very little is known about domains involved in GPCR-Jak interaction. It has been shown that the tyrosine residue within the conserved DRY motif and the C-terminal YIPP motif are important for Jak2 activation and association with certain GPCRs (Ali et al.,

1997; Mellado et al., 1998). The human PAFR contains a single tyrosine residue within its C-terminal tail. We next examined (Chapter V) the role of the receptor C-terminus in PAFR-mediated Jak2 activation using C-terminus deletion mutants K298Stop, T305Stop, C317Stop and V330Stop. The activation of Janus family members is hypothesized to occur as a consequence of auto- and/or trans-phosphorylation on tyrosine residues associated with cytokine-induced aggregation of the receptor chains and the associated Jaks. In many cases, regulation of catalytic activity by trans-phosphorylation occurs on residues within the enzyme activation loop (Pellegrini and Dusanter-Fourt, 1997; Feng et al., 1997). We, therefore, explored the possibility of regulation of Jak2 activity using a dominant negative mutant of Tyk2, K930I. In our study, we found that PAF induced Jak2 tyrosine phosphorylation in the presence of co-transfected K930I.

In summary, the results presented in this thesis examined the role of the Jak/STAT pathway in PAFR regulation and extended our current knowledge of mechanisms GPCR-Jak interactions.

III. ARTICLE

G-protein-independent activation of Tyk2 by the platelet-activating factor receptor.

Lukashova, V., Asselin, C., Krolewski, J.J., Rola-Pleszczynski, M., Stankova, J.

J. Biol. Chem. 276: 24113-24121, 2001

IV. ARTICLE

The C-terminus and Glu^{51} of platelet-activating factor receptor are critical for Tyk2-dependent PAFR promoter 1 activation

Lukashova, V., Chen, Z., Rola-Pleszczynski, M., Stankova, J.

Submitted

The C-terminus and Glu[51] of the Platelet-activating Factor Receptor are critical for Tyk2-dependent transcriptional activation .[1]

Viktoria Lukashova, Zhangguo Chen, Marek Rola-Pleszczynski and Jana Stankova*

From the Immunology Division, Department of Pediatrics, Université de Sherbrooke, Sherbrooke, Quebec, Canada J1H 5N4

Running Title: **PAFR and Tyk2 association**

* **To whom correspondence should be addressed**: Immunology Division, Faculty of Medicine, Université de Sherbrooke, 3001, North 12th Avenue, Sherbrooke (Quebec), Canada, J1H 5N4. Tel.: (819) 564-5268; FAX: (819) 564-5215;
email: Jana.Stankova@USherbrooke.ca.

Keywords: signal transduction, inflammatory mediators, tyrosine kinases, G-protein, G-protein-coupled receptor.

SUMMARY

Platelet-Activating Factor (PAF) activates the Jak/STAT pathway via its cognate receptor (PAFR), a member of the G-protein-coupled receptor superfamily (GPCR). We have recently reported that Tyk2 is constitutively associated with PAFR and is necessary for PAF-induced PAFR promoter 1 activation. In the present report, we determined the domains of PAFR important for Tyk2 association and activation. Our results showed that GST-fusion proteins, bearing the second and third intracellular loops or the C-terminus of PAFR, co-precipitated with both WT and a truncated Tyk2 (residues 1-601), whereas the the first intracellular loop of the receptor bound only the truncated kinase. PAFR mutants with deletions in the second intracellular loop, Δ131-133 and Δ127-133, showed 65% and 80% decrease in Tyk2 binding, respectively. On the other hand, the PAFR C-terminus deletion mutant T305Stop co-precipitated with Tyk2, indicating that the C-tail was not essential for Tyk2 binding. The regions necessary for Tyk2 activation were examined by using minigene constructs along with mutational analysis of the receptor. Constructs encoding the second or third intracellular loops or the C-terminus of the receptor inhibited PAFR promoter activation. Receptor mutations in the first, second and C-tail regions (L43F, G134D and M311Stop) were ~30-40% less effective than WT in Tyk2 activation. However, the PAFR mutant E51A bound Tyk2 similarly to WT PAFR, but failed to induce PAFR promoter activation, indicating that this residue was essential for Tyk2 activation. In summary, our results suggest that PAFR contains several binding sites for Tyk2, with the second intracellular loop being the most important. However, Tyk2-dependent transcriptional activation was dependent on the residue E51, in the first intracellular loop of the receptor, and the C-terminus.

INTRODUCTION

Platelet-activating factor (PAF) is a phospholipid originally identified as a mediator, released by sensitized rabbit basophils, which induced platelet aggregation. PAF is produced by a wide variety of cells and tissues including granulocytes, macrophages, endothelial cells, lung tissue, uterus, brain and kidney(1,2). In vivo administration of PAF can elicit symptoms of anaphylaxis, shock or immune complex disease. In addition, PAF can induce broncho-constriction, increased vascular permeability and decreased plasma volume, decreased cardiac output, extravasation of leukocytes as well as tissue necrosis (3-5). PAF is involved in the pathogenesis of allergic disorders, inflammation, anaphylactic shock, and various other physiological events (6-8). Recently, Shimizu and colleagues established transgenic mice overexpressing the PAF receptor (PAFR); these animals showed bronchial hyperreactivity, increased endotoxin lethality and melanocytic tumorigenesis (9). Conversely, PAFR knockout mice showed impaired anaphylactic responses (10). PAFR belongs to the G protein-coupled receptor (GPCR) superfamily and drives multiple signaling pathways, including the activation of phospholipases A2, C and D, activation of mitogen-activated protein kinases, phosphatidylinositol 3-kinase and multiple tyrosine kinases (6,7,11-13). One of the tyrosine kinases activated by PAF is Tyk2, which belongs to the Janus (Jak) family of kinases (14).

The Jak kinases represent a distinct family of cytosolic tyrosine kinases that have been implicated in the signal transduction of many members of the cytokine family (15). At present, the Jak family consists of four members, Jak1,2,3 and Tyk2. Jaks constitutively associate with intracellular domains of cytokine receptors and become

phosphorylated after ligand binding and aggregation of the respective receptor chains. Activated Jaks phosphorylate both the cytokine receptors and the Jaks themselves, thereby recruiting Signal Transducers and Activators of Transcriptions (STATs) and other signaling molecules into the activated receptor complex (16). STATs are then phosphorylated by Jaks, form homo- or heterodimers and translocate to the nucleus to set in motion target gene expression (17).

Although, for a long time, Jak/STAT signaling was considered as a unique feature of cytokine receptors, Marrero and colleagues provided the first evidence that the Jak/STAT pathway can be stimulated in rat aortic smooth muscle cells by a GPCR, the Angiotensin II AT1 receptor (18). Since, several groups, including ours, have shown that the Jak/STAT pathway can be activated by several GPCRs such as the chemokine receptors CCR2B, CCR5 and CXCR4 (19-21), and 5-HT2a receptors (22). Recently, we showed that the activation of Tyk2 by PAFR was G-protein-independent. In addition, PAFR co-immunoprecipitated with the full length Tyk2, as well as truncated Tyk2 mutants, indicating that at least two domains in the kinase, one in the JH6-JH7 region and the other in the JH3-JH5 region, are important for the binding to the receptor. In analogy to the cytokine receptors, PAFR and Tyk2 association was ligand-independent. PAFR also colocalized with Tyk2 in the myeloid cell line MonoMac-1 and COS-7 cells transfected with PAFR and Tyk2 cDNAs (14).

Tyk2 was originally cloned as an essential molecule for IFNα signaling (23,24), and it is also activated in response to several cytokines, including IL-6 (25), IL-10 (26), and IL-12 (27). Direct and specific interaction between IFNaR1 and Tyk2 has been demonstrated by co-immunoprecipitation of the two endogenous proteins (28) and by *in*

vitro binding of baculovirus-expressed Tyk2 with GST-fusion protein bearing a cytoplasmic domain of IFNaR1 (29). Later, it was shown that the Tyk2 binding domain is restricted to a 46-amino-acid membrane–proximal region of IFNaR1 with a particular importance of hydrophobic and acidic residues (30). In addition, direct association was observed between Tyk2 and the cytoplasmic region of the IL-12β1 receptor chain (31).

Mutational analysis clearly demonstrated the importance of membrane proximal domains of cytokine receptors in receptor/Jak interaction. The membrane-proximal region of IL-2R, encompassing 52 amino acids, was sufficient for Jak3 binding (32). A proline-rich motif, comprising approximately eight amino acids, termed box 1, was required for the interaction of Jak2 with the receptors for prolactin, erythropoietin, growth hormone, IL-6 and related cytokines which utilize the gp130 receptor subunit (33,34). GPCR-Jak interactions have not been extensively studied. However, the motif DRY in the second intracellular loop of CCR2B has been shown to be critical for Jak2 association (19) and the motif YIPP in the C-terminus of the AT1 receptor was required for the binding of the SHP-2 phosphatase, which in turns recruited Jak2 into the receptor complex (35).

Using PAFR GST-fusion proteins bearing receptor intracellular loops, as wel as PAFR deletion or substitution mutants, we investigated the regions of interaction between PAFR and the tyrosine kinase Tyk2.

EXPERIMENTAL PROCEDURES

Materials

PAF was from Cayman Chemical Co. (Ann Arbor, MI). WEB 2086 was from Boehringer Ingelheim GmbH (Ingelheim, Germany); *Pwo* polymerase and FuGENE6 transfection reagent were from Roche Molecular Biochemicals (Mississauga, ON, Canada), oligonucleotides were synthesized at Invitrogen (Burlington, ON, Canada), restriction endonucleases and modifier enzymes were from Promega (San Luis, CA) and Amersham Pharmacia Biotech. (Piscataway, NJ), bovine serum albumin, glutathione S agarose and protein A-Sepharose were from Sigma-Aldrich (St. Louis, MO). Antibodies used were rabbit polyclonal anti-Tyk2 and monoclonal anti-Tyr(P) (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-N-terminal region of Tyk2 (Transduction Laboratories, Mississauga, ON, Canada) and monoclonal anti-cMyc was form ATCC (clone 9E10, Manassas, VA). Horseradish peroxidase-conjugated goat anti-mouse and donkey anti-rabbit antibodies were from Jackson Immunoresearch (West Grove, PA). Human Tyk2 WT and mutant cDNAs were a kind gift from Dr. J.J. Krolewski (University of California, Irvine, CA) and described recently (30). Dr. G. Guillemette (Université de Sherbrooke, Sherbrooke, QC, Canada) kindly provided COS-7 cells. The PSP64 expression vector was from Promega.

Cell Culture and Transfection

COS-7 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (Bio Media Canada, Drummonville, QC, Canada) and 100 μg/ml garamycin. Cells were plated in

100-mm dishes (1 X 10^6 cells/dish) and transiently transfected with human cMyc-tagged PAFR cDNA, cloned into the pcDNA3 (Invitrogen), and hTyk2 cDNA or pcDNA3 (5 μg of DNA, total) using 10 μl of FuGENE according to the manufacturer's instructions. 48 h after transfection, cells were incubated without serum for 1 h and then stimulated with PAF (10^{-7} M) or left unstimulated. Cells were lysed and extracts used for immunoprecipitation.

Construction of Mutant Receptors

Single amino acid substitution mutants were constructed by PCR using cMyc-tagged PAFR cloned into the pSP64 expression vector as a template. We used sp6 primer as forward and the following oligonucleotides as reverse primers: F40G, 5'-ATGAAGATCTTTATCTCATTGAATTTCTTGCAAGGGTACAGGCGGGCACCGACCCACAG-3'; L43F, 5'-TGAATCTTTATCTCATTGAATTTCTTGCAAGGGTAAAA-GGCAAAGACCAA-3'; P45A, 5'-TGAAGATCTTTATCTCATTGAATTTCTTGCA-AGCGTACAGGCGGGC; C46W, 5'-CATGAGATCTTTATCTTATTGAATTTCTTC-CATGCGTACAGGCGGGC-3'; KK4748AA, 5'-CATGAAGATCTTTATCTCATTG-AATGCCGCGCAAGGGTACAGGCGG-GC-3'; F49V, 5'-CCATGAAGATCTTTAT-CTCGTTAACTTTCTTGCAAGG-3'; E51A, 5'-CATGAAGATCTTTATCGCATT-GAATTTCTT-3'; I52V, 5'-TGAAGATCTTTACCTCATT-GAATTTCTT. The mutant PCR products were digested with *HindIII-BglII* and replaced WT fragment from PAFR-pSP64 construct digested with the same restriction endonucleases. Mutated cMyc-tagged receptors were transferred from pSP64 to pcDNA3 using *HindIII* and *EcoRI* restriction endonucleases. Minigene constructs 1-in, 2-in, 3-in, encoding the 1st (from residue W38

to F55), 2nd (from residue N114 to G134) and 3rd (from residue V208 to L231) intracellular loops of PAFR were created by annealing of corresponding oligonucleotides: 1-in DIR, 5'-AATTCGCCGCCACCATGGCATGGGTCTTTGCCCGCCTGTACCCT-TGCAAGAAATTCAATGACATAAAGATCTTCTGAC-3' and 1-in-RVS, 5'-CCGG-GTCAGAAGATCTTTATCTCATTGAATTTCTTGCAAGGGTACAGGCGGGCAAA ACCCATCCCATGGTGGCGGCG-3'; 2-in DIR, 5'-AGCTGCCGCCACCATGGG-AAACCGCTTCCAGGCAGTAACTCGGCCGATCAAGACTGCTCAGGCCAACACC CGCAAGCGTGGCTGAG-3' and 2-in RVS, 5'-GATCCTCAGCCACGCTTG-CGGGTGTTGGCCTGAGCAGTCTTGATCGGCCGAGTTACTGCCTGGAAGCGGT TACCCATGGTGGCGGCA-3'; 3-in DIR, 5'-AGCTTGCCGCCACCATGGGAGT-CATCATCCGTACCTTGCTCATGCAGCCGGTGCAGCAGCAGCGCAACGCTGTC AAGCGCCGGGCGCTGTGAG-3' and 3-in RVS, 5'-GATCCTCACAGCGCCCGGC-GCTTCAGAGCGTTGCGCTGCTGCTGCACCGGCTGCATGAGCAAGGTACGGAT GATGACTCCCATGGTGGCGGCA-3'. Annealing products encoding the 2nd and 3rd intracellular loops of PAFR (2-in and 3-in) were ligated into pcDNA3 vector digested with *HindIII* and *BamHI* restriction endonucleases. Annealing product 1-in was at first treated with *Klenow* in the presence of GTP and CTP and then ligated into pcDNA3, digested with *EcoRI* and *EcoRV* restriction endonucleases. Deletion mutant Δ46-60 was constructed by removal of an internal *NcoI* fragment from the mutant C46W pSP64, followed by self ligation and transfer into the pcDNA3 vector using *HindIII* and *EcoRI* restriction enzymes. To construct deletion mutants Δ41-48 and Δ45-48, PCR products were generated using PAFR mutant F49VpSP64 as template, sp6 primer as forward and the following oligonucleotides as reverse primers: Δ41-48, 5'-AACGGCAAAGACCCA-

CAGCACGTA-3' and Δ45-48, 5'-AACGTACAGGCGGGCAAAG-ACCCA-3'. PCR products were then digested with HindIII and subcloned into F49V pSP64 digested by *HindIII* and *HpaI*. The fragment with the deletion was then introduced into the cMyc-tagged receptor coding sequence using *HindIII* and *Bst*EII restriction enzymes. G134D substitution was created by PCR using PAFR pSP64 as template, sp6 primer as forward and the oligonucleotide: 5'-CCACCCAGATGACCAAGGACAAAGAGATATCAC-CTTGCGGGTGTT-3' as reverse complement. The PCR product, digested with *HindIII*, was introduced into PAFR-pSP64 digested with *HindIII* and *MscI* restriction enzymes. The fragment with the substitution then replaced the *HindIII, BstEII* fragment in WT PAFR pcDNA3. Mutants with deletions in the 2nd intracellular loop were generated using G134D pcDNA3 as template, T7 primer and the following oligonucleotides: Δ131-133, 5'-ATCGGTGTTGGCCTGAGCAGTCTT-3'; Δ127-133, 5'-ATCAGCAGTCTTGAT-GGGCCGAGT-3'; Δ124-133, 5'-ATCGATGGGCCGAGTTACTGCCTG-3'; Δ121-133, 5'-ATCAGTTACTGCCTGGAAGCGGTT-3'; Δ118-133, 5'-ATCTGCCTGGAAGCG-GTTATAAGT-3'. PCR products digested with *HindIII* were then introduced into *HindIII, EcoRV* sites of G134D pcDNA3. PAFR promoter 1 reporter gene construct p0.16Luc (14) and PAFR mutants M311Stop and T305Stop were described previously (36). Constructs were sequenced at the University of Calgary, Alberta, Canada.

GST Fusion Constructs and in vitro binding

The GST-fusion protein bearing the 1st intracellular loop of PAFR was created by digestion of minigene 1-in pcDNA3 construct and pGEX-4T1 (Amersham Pharmacia Biotech Inc.) vector with *EcoR1* and *XhoI* restriction endonucleases followed by ligation

of purified vector and insert. Fusion protein bearing the 2nd and 3rd intracellular loops of the receptor (2,3-GST) was made by digestion of construct pAS2-1-123, encoding the 1st, 2nd and 3rd intracellular loops of PAFR with *StuI* and *SalI* followed by ligation of the fragment with vector pGEX-4T1 digested with *SmaI* and *XhoI* restriction endonucleases. C-tail-GST was generated by PCR using the following primers: 5'-CAGGAATTCACCAAGAAGT-TCCGCAAG-3' and 5'-GTAGTCGACTAATTTT-TGAGGGAATT-3'. PCR product was digested with *EcoRI* and *SalI* restriction enzymes and ligated into PGEX-4T1 vector digested with *EcoRI* and *XhoI* restriction endonucleases.

DH5α *E. coli* transformed with the resulting plasmid were grown at 37°C in TB supplemented with 50 μg/ml ampicillin to log-phase. Expression of the fusion protein was induced with 1 mM isopropyl-1-thio-D-galactopyranoside for 5 h at 37°C. Bacteria were lysed in lysis buffer (50 mM Tris, pH 7.5, 100 mM KCl, 1 mM EDTA, 2 mM $MgCl_2$, 20μM/ml of lysozyme, 1% of IGEPAL) on ice 30 min. After 20 min of centrifugation at 13000g, supernatants containing GST-fusion proteins were purified from the lysate using glutathione-agarose beads and visualized by Coomassie staining after SDS-PAGE. Each freshly purified recombinant protein was incubated 2h at 4°C with the lysate from COS-7 cells transfected with 3 μg of either WT Tyk2 or 1-601 deletion mutant Tyk2. Beads were pelleted and washed four times with lysis buffer. Bound proteins were eluted with Laemmli sample buffer, 30 min at room temperature, separated by SDS-PAGE and subjected to Western blot analysis.

Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting were performed as described previously (23). Briefly, 48 h after transfection, COS-7 cells were lysed in buffer: 50 mM Tris pH 7.5,1mM EGTA, 150 mM NaCl, 1 mM NaF, 1 mM Na_3VO_4, 1% NP-40, 1 mM PMSF, 0.25% sodium deoxycholate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin (Sigma), 30 min on ice. Lysates were incubated with anti-cMyc antibodies overnight at 4^0C. Immunoprecipitated proteins were separated on 10% SDS-PAGE and transferred to PVDF membranes. Blocking was performed in Tris-buffered saline (TBS) with 2.5% gelatin for 1 h and incubation with corresponding primary antibody in TBS-Tween 0.1% + 0.5% gelatin overnight at 4^0C. An enhanced chemiluminescent detection system was used for protein detection (Amersham).

Flow Cytometry Studies

COS-7 cells transfected with cMyc-tagged WT hPAFR or mutant receptor cDNA were harvested 48 h after transfection, and 2.5 x 10^5 cells were subjected to flow cytometry analysis using anti-cMyc antibody and fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (BD Biosciences, Mississauga, ON). Analysis was performed on a FACScan flow cytometer (BD Biosciences, Oakville, ON, Canada)).

Radioligand Binding Assay

Competition binding curves were done on COS-7 cells expressing the WT and mutant receptor species. Cells were harvested and washed twice in Hepes-Tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 12 mM $NaHCO_3$, 5.6 mM D-glucose, 0.49

mM $MgCl_2$, 0.37 mM NaH_2PO_4, 25 mM Hepes, pH 7.4) containing 0.1% (w/v) bovine serum albumin (BSA). Binding reactions were carried out on 5 x 10^4 cells in a total volume of 0.25 ml in the same buffer with 10 nM [^{3}H]-WEB2086 (PerkinElmer Life Science Products, Boston, MA) and increasing concentrations of nonradioactive WEB2086 or PAF for 90 min at 25°C. Reactions were stopped by centrifugation. The cell-associated radioactivity was measured by liquid scintillation.

Luciferase assay

Luciferase assay was performed as described previously (23). COS-7 cells were seeded in 24-well plates at a density 1x10^4/well and transfected with PAFR or mutant receptor cDNA (0.1μg/well), Tyk2 (0.05μg/well) or pcDNA3 and PAFR promoter construct p0.16Luc (0.1μg/well) cDNAs. When minigene constructs were used as competitors, total amount of DNA was 0.7 μg. 40 h after transfection cells were preincubated 1 h in medium without fetal bovine serum, stimulated with PAF (10^{-7}M) for 6 h, harvested and assayed for luciferase activity.

RESULTS

We have recently shown that PAFR associates with Tyk2 and Tyk2 truncated mutants, independently of ligand binding (14). In the present study, we investigated the PAFR regions involved in Tyk2 interaction. We produced GST-fusion proteins bearing different intracellular loops or the C-tail (46 residues) of the receptor. We then performed in vitro binding experiments with GST-PAFR fusion proteins and WT Tyk2 or a Tyk2 mutant containing the JH7-JH3 regions of the kinase (residues from 1 to 601), transiently expressed in COS-7 cells. The GST-PAFR proteins bound to glutathione-agarose beads were incubated with COS-7 cell lysates and analyzed by Western blot with anti-Tyk2 antibodies. Figure 1A shows that the GST-fusion protein, corresponding to the first intracellular loop of the receptor, was able to bind only the truncated kinase. However, the GST-fusion protein bearing the second and third intracellular loops or the C-tail of the receptor immunoprecipitated WT, as well as truncated Tyk2 (Figure 1B and 1C). Our results indicate that each intracellular loop, as well as the receptor C-tail contain a Tyk2 binding site.

We then examined the role of the C-terminus of PAFR in Tyk2 binding by using C-terminal deletion mutants of the receptor. COS-7 cells were transiently transfected with cMyc-tagged PAFR or T305Stop, a PAFR truncated mutant, and Tyk2 cDNAs. Cellular lysates were subjected to immunoprecipitation with anti-cMyc antibodies and protein complexes were analyzed by Western blotting with anti-Tyk2 antibodies. Data shown in Figure 2 indicate that Tyk2 bound to the truncated receptor, suggesting that the C-terminus of PAFR was not essential for kinase binding. Similar results were observed with the C-terminal deletion mutant M311Stop (data not illustrated). The membrane was

reblotted with anti-cMyc antibodies to verify PAFR expression in all samples (lower panel). These data indicate that although Tyk2 is able to bind to the C-terminus of the PAFR, this region is not necessary for binding to the receptor, in the presence of the other intracellular regions.

Our previous data showed that Tyk2 is necessary for PAF-stimulated PAFR promoter 1 activation (14). To further characterize this association, we generated minigene constructs encoding the PAFR intracellular loops and C-tail, then investigated their capacity to inhibit Tyk2-dependent PAFR transcription. COS-7 cells were transiently transfected with WT PAFR, reporter gene p0.16Luc and Tyk2 cDNAs. The minigene constructs, encoding the C-terminus of the receptor and the second intracellular loop, blocked PAF-induced PAFR promoter 1 activation when co-expressed with the receptor and Tyk2 in COS-7 cells ($p<0.05$, Figure 3A). The expression of the first intracellular loop had no effect. Similar inhibition was observed when two minigenes were co-expressed, although interestingly, a more profound inhibition was detected when the first and third receptor intracellular loops were co-expressed. Comparative statistical analysis showed a significant difference ($p<0.05$) between cells transfected with the minigene encoding the third intracellular loop (3-in) and mixture of two minigenes (1-in + 3-in). The expression of the minigenes had no effect on PAFR-Tyk2 binding, except for the intriguing finding of an increase in PAFR-Tyk2 association in the presence of the minigene encoding the first intracellular loop (Figure 3C and data not illustrated). In addition, inositol phosphate production, after PAF stimulation, was not modulated by minigene co-expression with PAFR in COS-7 cells, indicating that the minigenes did not affect this particular signaling pathway of the receptor (Figure 3D).

Previously, we have reported that the first intracellular loop of PAFR has a certain degree of homology with the IFNaR1 receptor region from residue 480 to 510 (37). This region consists of important domain(s) for Tyk2 binding (38). Our model predicted that the first intracellular loop of the receptor might interact with two regions in Tyk2, spanning residues 29-35 and 196-213. Interestingly, the latter region appeared also to interact with the second intracellular loop of the receptor. Based on our theoretical prediction, we made a series of PAFR deletion mutants and single amino acid substitution mutants to study the role of the first and the second intracellular loops of PAFR. Figure 4 indicates the localization of the substitution and deletion mutants used in this study. Ligand binding and cell surface expression were evaluated after transfection of WT PAFR and mutant receptor cDNA in COS-7 cells. Our data suggests that deletion of several residues in either the first or second intracellular loop had a profound effect on ligand-binding capacity of the receptor. The deletion mutants in the first intracellular loop (Δ41-48 and Δ46-60) resulted in receptors which were expressed on the cell surface (as determined by FACS analysis) but did not bind ligand (results not illustrated). However, the mutants with a deletion in the second intracellular loop (Δ127-133 and Δ131-133), had higher affinity for ligand than WT receptors (K_i values of 5.33 nM and 9.0 nM respectively, vs 34.1 nM for WT) (Table I). On the other hand, ligand-binding sites on the cell surface were reduced, compared to WT. Two other mutant receptors, G134D and KK47,48AA also displayed higher affinity for WEB2086, with respective K_i values of 13.9 and 6.4 nM. No significant difference was found in the affinity for WEB2086 between the other mutants and the WT PAFR (Table I). The expression on the cell surface of deletion mutants in both the first and second intracellular loop (Δ41-

48, Δ46-60, Δ127-133 and Δ131-133) was similar to the WT, as measured by cytofluorimetry, however these receptors were unable to induce inositol phosphate production upon PAF stimulation, while IP production stimulated by all the other mutants was similar to that of WT PAFR (data not illustrated).

Next, we studied the capacity of Tyk2 binding by the deletion mutants, using COS-7 cells transfected with Tyk2 and WT PAFR or deletion mutant cDNAs. Forty-eight hours after transfection, the cells were lysed and lysates were subjected to immunoprecipitation with anti-cMyc antibodies followed by Western blotting with anti-Tyk2 antibodies. We observed a decrease of 50%, 65% and 80% in Tyk2 binding with deletion mutants Δ41-48, Δ127-133 and Δ131-133, respectively. Conversely, PAFR deletion mutant Δ46-60 demonstrated a 3-fold increased binding of the kinase (Figure 5). All the other receptor substitution mutants bound Tyk2 similarly to WT PAFR (data not shown).

To further characterize the interaction of Tyk2 with the mutant receptors, we measured their capacity to induce PAFR promoter 1 activation following PAF stimulation. COS-7 cells were transiently transfected with the WT or mutant receptors, reporter gene p0.16Luc and Tyk2 cDNAs. Deletion mutant Δ127-133 showed reduced capacity to induce PAFR promoter activation ($p<0.05$, Figure 6A), in agreement with the lower binding capacity for Tyk2. Deletion of the 30 C-terminal residues in M311Stop resulted in a mutant receptor that was 40% less effective compared to WT in the induction of promoter activation, suggesting that the C-terminus of PAFR contains an important domain for the activation of Tyk2 function.

Sequence alignment of PAF receptors form rat, guinea pig and human indicates that almost all the amino acids in the first intracellular loop as well as a cluster of residues from 130 to 135 are conserved between the three species (Bito and Shimizu, 1997). Changes in either the basic (KK4748AA, G134D) environment or substitution of L43 for phenylalanine diminished Tyk2-dependent signaling ($p<0.05$, Figure 6B). Substitution of the hydrophobic residues F40 and F49 for glycine or substitution of isoleucine at position 52 for a valine residue did not change the capacity of mutant receptors to induce promoter activation. The first intracellular loop of the PAFR consists of sequence LYPCKK that has some degree of resemblance to the Box-1-like motif of interferon receptors. Mutation of P45 to alanine resulted in a 40% increase ($p<0.05$) in luciferase activity, possibly allowing for more accessibility of signaling molecules. Finally, mutation of E51 to alanine completely abolished Tyk2 signaling ($p<0.001$), indicating that this residue is of critical importance for PAF-induced Tyk2-dependent transcriptional activation of PAFR promoter (Figure 6B). The positive charge of this residue may be important since the substitution of the neighboring isoleucine (I52) or hydrophobic valine, did not alter Tyk2-dependent PAFR transcription.

DISCUSSION

In this report, we show that the activation of Tyk2 required a restricted number of residues in PAFR, in particular the glutamic acid at position 51, but the association of the kinase with PAFR involved several of the intracellular regions of the receptor. The AT1 and B1 receptors have also been shown to activate Tyk2, but the association of Tyk2 with GPCRs has only been studied with the AT1 receptor (18,39). Ali and colleagues showed that Tyk2, in contrast to Jak2, does not associate with the AT1 receptor, either constitutively or in a ligand dependent manner (40). In the case of Jak2, its association with AT1 is dependent on a four residue motif YIPP in the C-terminus, both for association and activation, although the association has shown to be indirect and via the SHP-2 phosphatase (35,40). Only one other study examined the association of GPCRs and Jaks: the DRY motif in the second intracellular loop, which had been shown to be important in G-protein activation, was essential for Jak2 association with the CCR2 receptor (19). Both these reports stressed the importance of the tyrosine residue for the association. In the PAFR, only two tyrosines are found in the intracellular regions of the receptor, one in the first intracellular loop (Y46) and the other in the C-terminal tail (Y309) and neither is essential for Tyk2 association since deletion mutants lacking these residues (Δ41-48 and T305Stop) were able to bind Tyk2.

In the case of both the AT1 and CCR2 receptors, the association is ligand-dependent (19,40). Moreover, with CCR2, Jak2 activation seems to be necessary for subsequent Gi association and activation (19). This is in contrast with the PAFR, where Tyk2 association is ligand-independent and activation is G-protein-independent (14). Stimulation with PAF does not modulate the co-localization or quantity of Tyk2 co-

immunoprecipitated with PAFR (14). The association of Jak kinases with cytokine receptors has been shown to be ligand-independent. Colamonici and colleagues established that there is specific constitutive interaction between IFNaR1 and Tyk2, both *in vitro* (29) and *in vivo* (28). Using EGFR/IL-12R chimeras, it was reported that the β1 subunit of IL-12R associated with Tyk2, whereas the β2 subunit interacted with Jak2, and kinase-receptor association did not quantitatively change after ligand stimulation (31).

Our study demonstrated that there are multiple binding sites on the PAFR for Tyk2, again, in contrast, to what was shown with the AT1 and CCR2 receptors. We observed binding of Tyk2 by GST-fusion proteins containing the intracellular loops and the C-terminus. Interestingly, the GST-fusion protein encoding the first intracellular loop could not bind the WT Tyk2 protein although it bound the deletion mutant Tyk2 1-601, suggesting that binding by the other regions of the PAFR may modulate the structure of the kinase to permit binding to the first intracellular loop. Our results also show that the C-terminus can bind the kinase, but this region of the receptor is not obligatory for the association, as a truncation mutant lacking most of the C-tail (T305Stop) can immunoprecipitate Tyk2. However, the C-terminus plays a determinant role in Tyk2 activation, since a minigene encoding the C-terminus inhibited Tyk2-dependent PAFR gene transcription and the C-terminus deletion mutant M311Stop failed to activate the PAFR promoter. The multiple binding sites on PAFR are in contrast with the restricted domains identified in the AT1 and CCR2 receptors, where the mutation of the respective tyrosine residues abolished both the kinase association and activation (19,40). Previously, we reported that a truncated Tyk2, containing only the JH6 and JH7 region (1-262) or only the JH3-JH5 region (263-601) can associate with PAFR (14). These

results are also consistent with the notion of multiple binding sites critical for the PAFR-Tyk2 interaction.

In a number of cytokine receptors, a proline-rich box 1 motif was shown to be important in Jak-receptor association (33,34). However, in most cases studied in details, box 1 sequence alone was not sufficient for Jak kinase binding. For example, in the case of gp130, a 26-amino acid region which included the Box 1 motif was needed for Jak co-immunoprecipitation (41). In the GM-CSF receptor β chain, Box 1 and the following 14 residues were necessary for binding of Jak2 (42). The Tyk2 binding domain was shown to localize in the IFNaR1 cytoplasmic region, spanning residues 465 to 511 with the most critical interaction occurring between residues 486 and 511 (38). The first intracellular loop of PAFR has a certain degree of homology with an IFNaR1 region of residues 480 to 510 (43). However, deletion of residues from 41 to 48 in the first intracellular loop of the PAF receptor resulted only in partial inhibition of Tyk2 co-immunoprecipitation, suggesting that the other receptor regions might be more pertinent for kinase binding. Interestingly, a more profound inhibition was observed when basic residues (131-133) in the second intracellular loop of PAFR were deleted. Taken together with the results showing that the minigene encoding the second intracellular loop also had the strongest effect in inhibiting PAFR gene promoter activation, this would indicate that the second loop was the most pertinent site for PAFR-Tyk2 interaction. These findings are in contrast to the requirement for hydrophobic and acidic residues for the IFNaR1-Tyk2 interaction (38).

Site-directed mutagenesis of cytokine receptors indicates that the binding domain for Janus kinases is also necessary for cytokine-induced signaling. Substitution of a leucine or an isoleucine cluster to alanines resulted in a lack of Tyk2 binding to IFNaR1 and an inhibition of IFNα-induced Tyk2 and STAT2 phosphorylation. Point mutation at residues L306 or E307 in a conserved LEVL motif of the EPO receptor resulted in an impaired STAT phosphorylation and induction of early response genes (37). In contrast, mutation of a single residue in PAFR can disrupt one function whereas other functions remain unaffected. Recently, our group reported that the PAFR mutant Y293A, which could not couple to G-proteins but could activate Tyk2-mediated transcriptional activation, was still capable of mediating arrestin translocation and co-immunoprecipitation (14,36). The activation capacity of our deletion mutants in the first intracellular loop could not be verified since these receptors, although well expressed on the cell surface, did not bind the ligand. Extensive mutational analysis did not show any changes in receptor-Tyk2 association, when one or two residues were changed. However, changes in the capacity of Tyk2 activation were found, with the glutamic acid at position 51 being essential for Tyk-dependent transcription of PAFR promoter, whereas other receptor functions remained unchanged. Reduced PAFR transcription was also observed in case of G134D, KK4748AA and L43F mutants. It is not unreasonable to suggest that these substitutions change receptor conformation in such a way that Tyk2 activation but not association, is modified.

We have shown that PAF can stimulate the phosphorylation of several STAT proteins (14), but at present, it is not known whether PAFR directly interacts with the STATs. However, we could hypothesize that the E51A substitution occurs nearby the receptor

STAT-binding site. For example, mutations of residues in close proximity to the STAT2 binding site on IFNaR1 affect significantly the co-immunoprecipitation of the receptor with STAT2 and IFN-α-mediated transcription (44). It will therefore be interesting to investigate the potential PAFR-STAT interactions.

In summary, we have shown, in the present study, that PAFR has multiple binding sites for Tyk2. The kinase can bind all the intracellular regions of the receptor, but the second intracellular loop is necessary for productive association of Tyk2 with PAFR. The second and third intracellular loops along with the C-terminus contribute to the productive activation of Tyk2, with the E51 residue in the first intracellular loop being essential for Tyk2-dependent PAFR gene expression.

REFERENCES

1. Snyder, F. (1985) *Med Res Rev* **5**, 107-140

2. Hanahan, D. J. (1986) *Annu Rev Biochem* **55**, 483-509

3. Doebber, T. W., Wu, M. S., and Biftu, T. (1986) *J Immunol* **136**, 4659-4668

4. Pretolani, M., Lefort, J., Malanchere, E., and Vargaftig, B. B. (1987) *Eur J Pharmacol* **140**, 311-321

5. Sun, X. M., and Hsueh, W. (1991) *J Immunol* **147**, 509-514

6. Prescott, S. M., Zimmerman, G. A., and McIntyre, T. M. (1990) *J Biol Chem* **265**, 17381-17384

7. Izumi, T., and Shimizu, T. (1995) *Biochim Biophys Acta* **1259**, 317-333

8. Bazan, N. G., Zorumski, C. F., and Clark, G. D. (1993) *J Lipid Mediat* **6**, 421-427

9. Ishii, S., Nagase, T., Tashiro, F., Ikuta, K., Sato, S., Waga, I., Kume, K., Miyazaki, J., and Shimizu, T. (1997) *Embo J* **16**, 133-142

10. Ishii, S., Kuwaki, T., Nagase, T., Maki, K., Tashiro, F., Sunaga, S., Cao, W. H., Kume, K., Fukuchi, Y., Ikuta, K., Miyazaki, J., Kumada, M., and Shimizu, T. (1998) *J Exp Med* **187**, 1779-1788

11. Liu, B., Nakashima, S., Kanoh, H., Takano, T., Shimizu, T., and Nozawa, Y. (1994) *J Biochem (Tokyo)* **116**, 882-891

12. Honda, Z., Takano, T., Gotoh, Y., Nishida, E., Ito, K., and Shimizu, T. (1994) *J Biol Chem* **269**, 2307-2315

13. Stephens, L., Jackson, T., and Hawkins, P. T. (1993) *J Biol Chem* **268**, 17162-17172

14. Lukashova, V., Asselin, C., Krolewski, J. J., Rola-Pleszczynski, M., and Stankova, J. (2001) *J Biol Chem* **276**, 24113-24121

15. Schindler, C., and Darnell, J. E., Jr. (1995) *Annu Rev Biochem* **64**, 621-651

16. Ihle, J. N. (1995) *Nature* **377**, 591-594

17. Darnell, J. E., Jr. (1997) *Science* **277**, 1630-1635

18. Marrero, M. B., Schieffer, B., Paxton, W. G., Heerdt, L., Berk, B. C., Delafontaine, P., and Bernstein, K. E. (1995) *Nature* **375**, 247-250

19. Mellado, M., Rodriguez-Frade, J. M., Aragay, A., del Real, G., Martin, A. M., Vila-Coro, A. J., Serrano, A., Mayor, F., Jr., and Martinez, A. C. (1998) *J Immunol* **161**, 805-813

20. Wong, M., Uddin, S., Majchrzak, B., Huynh, T., Proudfoot, A. E., Platanias, L. C., and Fish, E. N. (2001) *J Biol Chem* **276**, 11427-11431

21. Vila-Coro, A. J., Rodriguez-Frade, J. M., Martin De Ana, A., Moreno-Ortiz, M. C., Martinez, A. C., and Mellado, M. (1999) *Faseb J* **13**, 1699-1710

22. Guillet-Deniau, I., Burnol, A. F., and Girard, J. (1997) *J Biol Chem* **272**, 14825-14829

23. Krolewski, J. J., Lee, R., Eddy, R., Shows, T. B., and Dalla-Favera, R. (1990) *Oncogene* **5**, 277-282

24. Velazquez, L., Fellous, M., Stark, G. R., and Pellegrini, S. (1992) *Cell* **70**, 313-322

25. Stahl, N., Boulton, T. G., Farruggella, T., Ip, N. Y., Davis, S., Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Barbieri, G., Pellegrini, S., and et al. (1994) *Science* **263**, 92-95

26. Finbloom, D. S., and Winestock, K. D. (1995) *J Immunol* **155**, 1079-1090

27. Bacon, C. M., McVicar, D. W., Ortaldo, J. R., Rees, R. C., O'Shea, J. J., and Johnston, J. A. (1995) *J Exp Med* **181**, 399-404

28. Colamonici, O. R., Uyttendaele, H., Domanski, P., Yan, H., and Krolewski, J. J. (1994) *J Biol Chem* **269**, 3518-3522

29. Colamonici, O., Yan, H., Domanski, P., Handa, R., Smalley, D., Mullersman, J., Witte, M., Krishnan, K., and Krolewski, J. (1994) *Mol Cell Biol* **14**, 8133-8142

30. Yan, H., Piazza, F., Krishnan, K., Pine, R., and Krolewski, J. J. (1998) *J Biol Chem* **273**, 4046-4051

31. Zou, J., Presky, D. H., Wu, C. Y., and Gubler, U. (1997) *J Biol Chem* **272**, 6073-6077

32. Nelson, B. H., Lord, J. D., and Greenberg, P. D. (1996) *Mol Cell Biol* **16**, 309-317

33. Rane, S. G., and Reddy, E. P. (2000) *Oncogene* **19**, 5662-5679

34. Pellegrini, S., and Dusanter-Fourt, I. (1997) *Eur J Biochem* **248**, 615-633

35. Marrero, M. B., Venema, V. J., Ju, H., Eaton, D. C., and Venema, R. C. (1998) *Am J Physiol* **275**, C1216-1223

36. Chen, Z., Dupre, D. J., Le Gouill, C., Rola-Pleszczynski, M., and Stankova, J. (2002) *J Biol Chem* **277**, 7356-7362

37. Miura, O., Cleveland, J. L., and Ihle, J. N. (1993) *Mol Cell Biol* **13**, 1788-1795

38. Yan, H., Krishnan, K., Lim, J. T., Contillo, L. G., and Krolewski, J. J. (1996) *Mol Cell Biol* **16**, 2074-2082

39. Ju, H., Venema, V. J., Liang, H., Harris, M. B., Zou, R., and Venema, R. C. (2000) *Biochem J* **351**, 257-264

40. Ali, M. S., Sayeski, P. P., Dirksen, L. B., Hayzer, D. J., Marrero, M. B., and Bernstein, K. E. (1997) *J Biol Chem* **272**, 23382-23388

41. Tanner, J. W., Chen, W., Young, R. L., Longmore, G. D., and Shaw, A. S. (1995) *J Biol Chem* **270**, 6523-6530

42. Zhao, Y., Wagner, F., Frank, S. J., and Kraft, A. S. (1995) *J Biol Chem* **270**, 13814-13818

43. Lukashova, V., Lukashev, V.A., Lukashev, V.V., Rola-Pleszczynski, M., Stankova, J.,. (2000) *BGRS Proceedings* **2**, 204-206

44. Krishnan, K., Singh, B., and Krolewski, J. J. (1998) *J Biol Chem* **273**, 19495-19501

ACKNOWLEDGMENTS

The authors would like to acknowledge the excellent technical advice and collaboration of Denis Gingras and Sylvie Turcotte. We also thank Dr. C. Le Gouill for a critical review of the manuscript.

FOOTNOTES

This work was supported by a grant from the Canadian Institutes for Health Research and a scholarship to V.L. from the Ministère de l'éducation du Quebec.

To whom correspondence should be addressed: Immunology Division, Faculty of Medicine, Université de Sherbrooke, 3001, North 12th Avenue, Sherbrooke (Québec), Canada, J1H 5N4. Tel.: (819) 564-5268; FAX: (819) 564-5215; email: Jana.Stankova@USherbrooke.ca

The abbreviations used are: G-protein, GTP-binding regulatory protein; GPCR, G-protein-coupled receptor; Jak, Janus kinase; PAF, platelet-activating factor; PAFR, platelet-activating factor receptor; STAT, signal transducers and activators of transcription; IFN, interferon; IL, interleukin; JH, Jak homology;

FIGURE LEGENDS

Figure 1. Tyk2 binding to PAFR GST-fusion proteins. COS-7 cells were transfected with Tyk2 or truncated Tyk2 (amino acids from 1 to 601) cDNAs. Cellular lysates were incubated with GST-fusion proteins bearing the first (1-in) intracellular loop (A) or second and third (2,3-in) intracellular loops or C-terminal (C-tail) 46 residues of PAFR (B). Precipitated complexes were separated on SDS-PAGE, transferred to a PVDF membrane and analyzed by Western blot with anti-Tyk2 antibodies.

Figure 2. Binding of Tyk2 by PAFR C-terminal deletion mutants. COS-7 cells were transiently transfected with cMyc tagged-WT PAFR or a receptor deletion mutants (-T305Stop) and Tyk2 (or pcDNA3) cDNAs. 48 h after transfection, cells were lysed and immunoprecipitated with anti-cMyc antibodies. Immune complexes were separated on a SDS-PAGE, transferred to a PVDF membrane and analyzed by Western blot with anti-Tyk2 antibodies. PAFR expression was assessed by reprobing the membrane with anti-cMyc antibodies.

Figure 3. Minigene constructs encoding the intracellular loops of the receptor inhibit of PAFR promoter activation but not inositol phosphate production. COS-7 cells were transfected with PAFR and the luciferase reporter construct of the PAFR promoter (p0.16Luc). The following cDNAs were also co-transfected as indicated: Tyk2 (or pcNDA3), minigene constructs, encoding the first (1-in), or second (2-in), or third (3-in) intracellular loops of PAFR or C-terminal (c-tail) 46 residues (PAFR/competitor ratio = 1/5). **A.** individual minigenes were used as competitors. **B.** Combinations of two

minigenes were used to assess cooperativity. Fold induction of luciferase activity was measured after 6 h of stimulation by PAF (10^{-7}M). Values represent the averages of five experiments, each done in duplicate, ± S.E. * $p<0.05$, ** $p<0.01$. **C.** COS-7 cells were transfected with PAFR, Tyk2 or pcDNA3 (C) and minigenes encoding 1st (1-in) or 3rd (3-in) intracellular loops of PAFR (PAFR/competitor = 1/5). After immunoprecipitation with anti-cMyc antibodies, Western blotting was performed with anti-Tyk2 antibodies (top). PAFR protein loading was analyzed with anti-cMyc antibodies (bottom). **D.** COS-7 cells were transfected with PAFR and minigene constructs encoding intracellular loops or c-tail of PAFR (PAFR/competitor = 1/10). Results represent total inositol phosphate production after 10 min of PAF (10^{-6}M) stimulation.

Figure 4. Putative seven transmembrane domain topography of the PAF receptor. Solid circles indicate the location of residues which were mutated for this study, the substituting residues are illustrated. Location of deletion mutants are indicated by arrows.

Figure 5. Coimmunoprecipitation of Tyk2 with PAFR deletion mutants. COS-7 cells were transiently transfected with Tyk2 and WT PAFR or indicated mutant cDNAs. Immunoprecipitation and Western blotting was performed as described in Figure 1.

Figure 6. Decreased PAFR promoter activation by deletion and substitution PAFR mutants. COS-7 cells were transfected with WT PAFR or deletion mutant receptors (**A**), or single amino acid substitution mutant receptor (**B**), with reporter gene p0.16Luc and

Tyk2 (or pcDNA3) cDNAs. Results represent luciferase activity (fold induction) where the activity in unstimulated COS-7 cells transfected with WT PAFR, p0.16Luc and Tyk2 was normalized to 1. Values represent the averages of five experiments, each done in duplicate, ± S.E. * $p<0.05$, ** $p<0.001$.

TABLE 1

Receptor	B_{max}	WEB2086 K_i
WT	1674000 ± 134000	34.1 ± 1.8
F40G	1130000 ± 255000	35.2 ± 1.6
L43F	1525000 ± 115000	31.1 ± 1.1
P45A	1425000 ± 105000	27.8 ± 3.3
KKAA	173000 ± 28000	6.4 ± 1.2
F49V	960000 ± 120000	25.5 ± 1.3
E51A	2010000 ± 55000	37.1 ± 0.4
I52V	2070000 ± 120000	35.1 ± 0.5
G134D	480000 ± 10000	13.9 ± 0.7
Δ127-133	250000 ± 80000	5.33 ± 0.2
Δ131-133	955000 ± 285000	9.00 ± 0.2

Table I Ligand binding parameters of the WT and mutant hPAFRs

Binding parameters were determined as described under "Materials and Methods" on transiently transfected COS-7 cells. Receptor densities (B_{max}) are indicated in receptors/cell, and dissociation constants (K_i) are indicated in nM The results are mean ± S.E of three independent experiments, each done in duplicate.

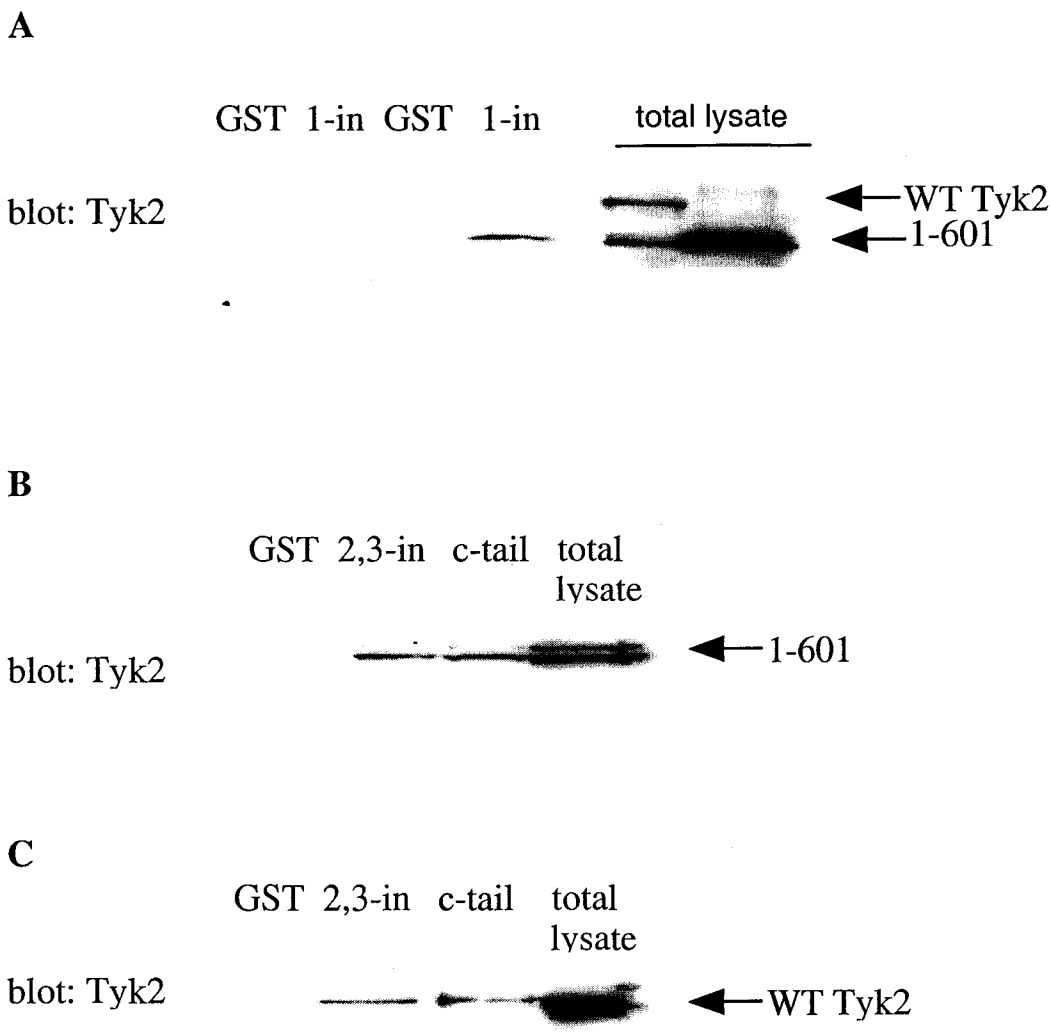

A
GST 1-in GST 1-in
total lysate
blot: Tyk2
WT Tyk2
1-601
B
GST 2,3-in c-tail total lysate
blot: Tyk2
1-601
C
GST 2,3-in c-tail total lysate
blot: Tyk2
WT Tyk2

Figure 1.

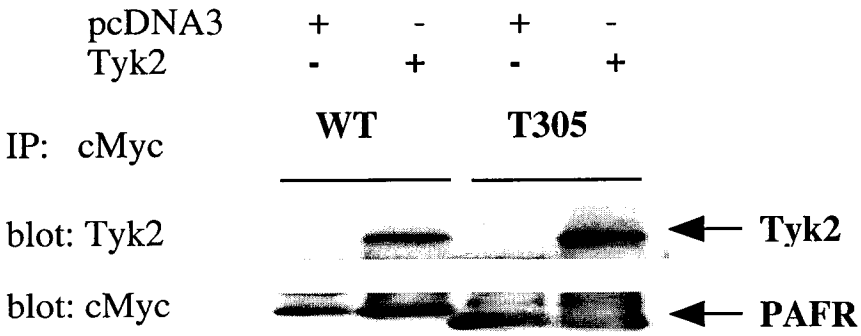
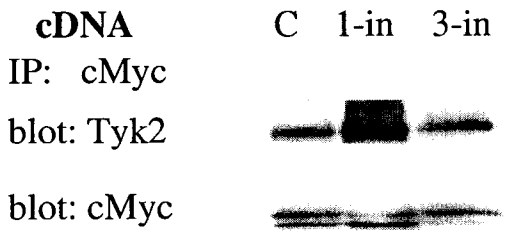


Figure 2.

C.


cDNA
C
1-in
3-in
IP: cMyc
blot: Tyk2
blot: cMyc

D.

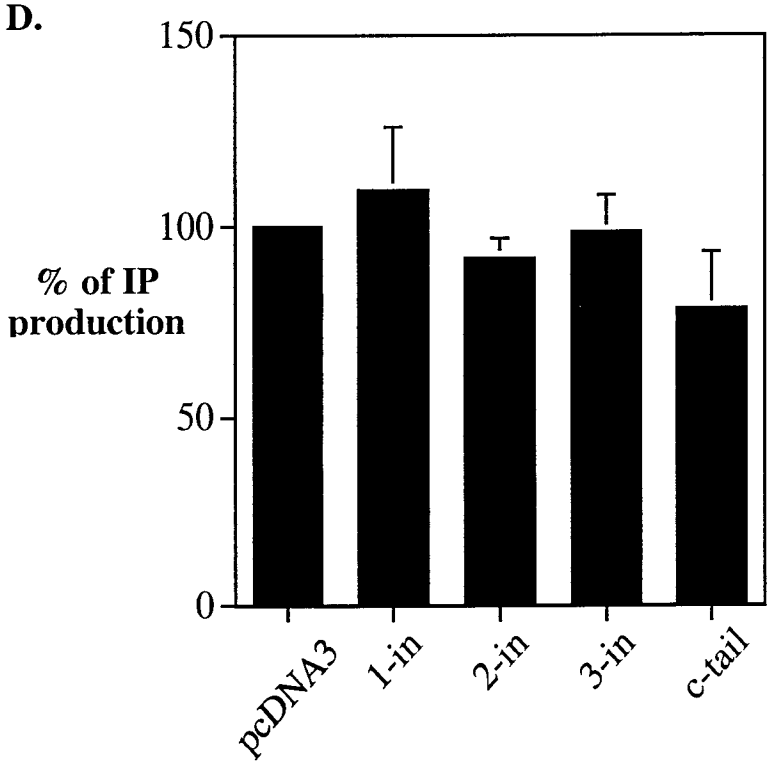

150
100
50
0
% of IP production
pcDNA3
1-in
2-in
3-in
c-tail

Figure 3.

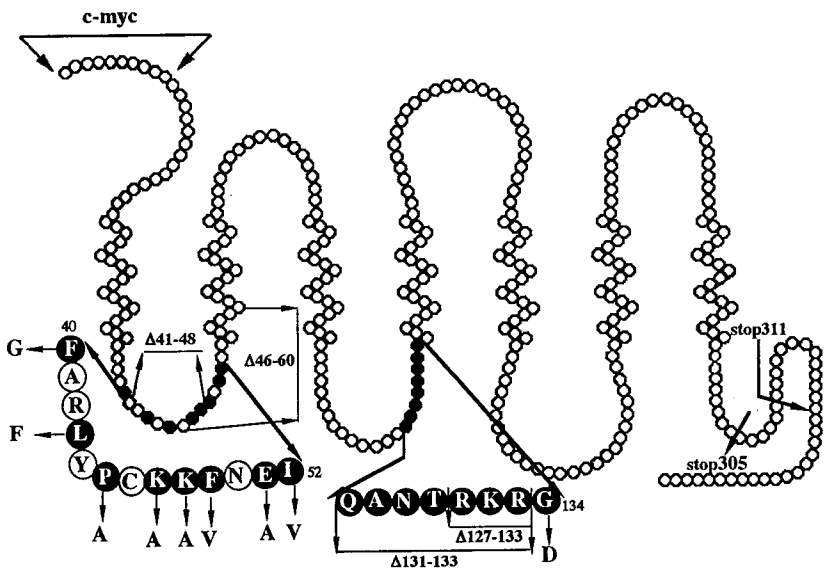

c-myc
40
G
F
A
R
F
L
Y
P C K K F N E I
52
A
A A V
A V
Δ41-48
Δ46-60
Q A N T R K R G
134
Δ127-133
Δ131-133
D
stop311
stop305

Figure 4.

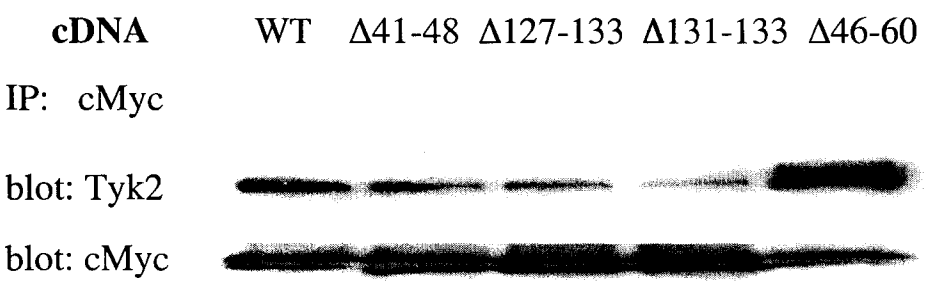
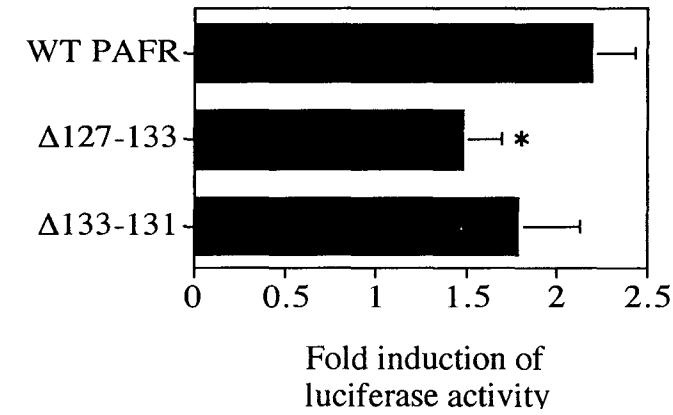

cDNA WT Δ41-48 Δ127-133 Δ131-133 Δ46-60
IP: cMyc
blot: Tyk2
blot: cMyc

Figure 5.

A.


WT PAFR
Δ127-133
Δ133-131
*
0
0.5
1
1.5
2
2.5
Fold induction of
luciferase activity

B.

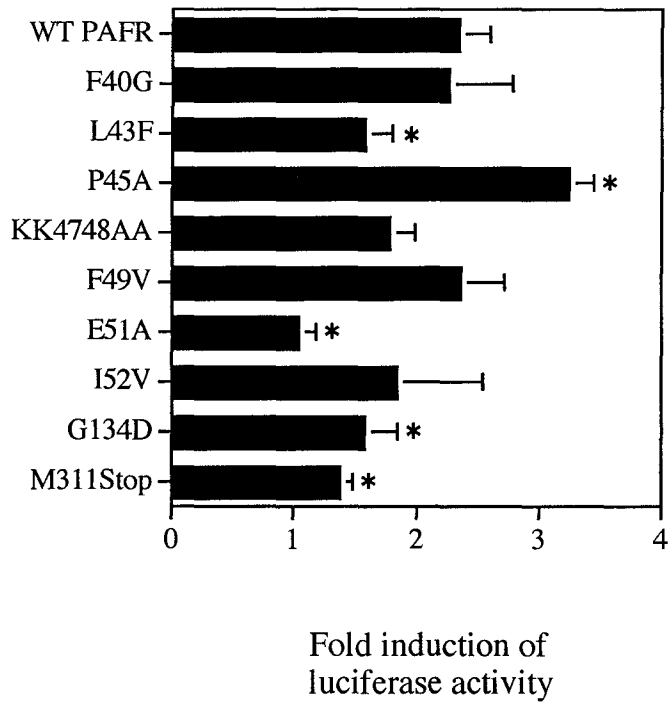

WT PAFR
F40G
L43F
P45A
KK4748AA
F49V
E51A
I52V
G134D
M311Stop
*
0
1
2
3
4
Fold induction of
luciferase activity

Figure 6.

V. ARTICLE

Jak2 activation by platelet-activating factor receptor: role of Tyk2 and PAFR C-terminus.

Lukashova, V., Chen, Z., Duhe, R., Rola-Pleszczynski, M., Stankova, J.

Submitted

Jak2 activation by the platelet-activating factor receptor: Role of Tyk2 and PAFR C-terminus[1].

Viktoria Lukashova[†], Zhangguo Chen[†], Roy J. Duhé[‡], Marek Rola-Pleszczynski[†] and Jana Stankova[†]*

[†]Immunology Division, Department of Pediatrics, Université de Sherbrooke, Sherbrooke, Quebec, Canada J1H 5N4 and [‡]Department of Pharmacology and Toxicology, University of Mississippi, MS, U.S.A.

Running Title: **Jak2 activation by PAF**

[1]* **To whom correspondence should be addressed**: Immunology Division, Faculty of Medicine, Université de Sherbrooke, 3001, North 12th Avenue, Sherbrooke (Quebec), Canada, J1H 5N4. Tel.: (819) 564-5268; FAX: (819) 564-5215;
email: Jana.Stankova@USherbrooke.ca.
The authors wish to acknowledge support from the Canadian Institutes for Health Research. V.L. is a recipient of a scholarship from the Ministère d'Éducation du Québec, R.J.D. is the recipient of a Research Scholar Grant (RSG-01-060-01-CDD) from the American Cancer Society and M. R.-P. is the recipient of the Canada Research Chair in Inflammation.

Keywords: human monocytes/macrophages, lipid mediators, signal transduction, inflammation, tyrosine kinases, G-protein-coupled receptor, Jak/STAT.

Abstract

Platelet-activating factor (PAF) is a phospholipid with multiple physiological and pathological actions. The PAF receptor (PAFR) belongs to the G-protein-coupled, heptahelical receptor superfamily. Recently, we have shown that PAF signals through the Jak/STAT pathway and that Tyk2 plays an essential role in PAF-induced PAFR promoter 1 activation. In the present study, we found that PAF stimulated Jak2 tyrosine phosphorylation in the monocytic cell line MonoMac-1, as well as in COS-7 cells transfected with PAFR and Jak2 cDNAs. The use of a G-protein-uncoupled PAFR (D289A) mutant indicated that Jak2 activation was G-protein independent. Interestingly, following PAF stimulation, Jak2 co-immunoprecipitated with PAFR in the presence of active Tyk2 but not with a kinase-inactive Tyk2 mutant, K930I. Moreover, Tyk2-K930I completely blocked PAF-stimulated Jak2 phosphorylation. Gradual deletion of C-terminal residues of the PAFR resulted in progressively decreased Jak2 activation. Deletion of twelve C-terminal residues in mutant V330Stop diminished Jak2 tyrosine phosphorylation by 17%. Further deletions of 25 to 37 residues form the PAFR C-tail (C317Stop, M311Stop and T305Stop) resulted in a 50% decrease of Jak2 phosphorylation compared to the WT receptor. The complete removal of the C-tail resulted in a mutant (K298Stop) which failed to activate Jak2, suggesting that the receptor C-terminal region contains important domains for Jak2 activation. Finally, the co-expression of a minigene encoding the C-terminus of PAFR, partially inhibited PAF-induced kinase activation. Taken together, our results indicate that PAF activates Jak2 and that Tyk2 and the C-terminal tail of PAFR are of critical importance for PAF-induced Jak2 activation.

Introduction

Platelet-activating factor (PAF) is a potent phospholipid mediator with diverse physiological and pathological effects which plays an important role in allergic disorders, inflammation and in the physiology of reproductive, cardiovascular and central nervous systems (1, 2). It is produced not only by monocytes, but also by neutrophils, basophils, eosinophils, mast cells, lymphocytes, platelets, endothelial cells and fibroblasts (3, 4). Many of these cells which produce PAF, as well as other cell types, such as smooth muscle cells and neurons, can become targets of PAF bioactions. In spite of its short half-life *in vivo*, PAF can produce sustained inflammatory reactions. For instance, inhalation of PAF by humans results in an acute bronchospasm that resolves within an hour and is followed by airway hypersensitivity to methacholine that may persist for several weeks (5). Injected PAF causes an immediate wheal and flare reaction and a later erythema and induration that can persist for more than 36 hours (6). The prolonged biological effects of PAF may result from the production of a number of cytokines, such as IL-1, TNF, IL-6 and IL-8 (7-12). Furthermore, PAF can prime cells for enhanced responsiveness to a second stimulus in terms of cytokine production (13, 14).

PAF exerts its effects via a specific receptor that is a member of the G-protein coupled receptor superfamily (GPCR). The type of G-proteins involved in PAF responses may differ according to cell type. In macrophages and neutrophils, PAF-induced phosphoinositide turnover is sensitive to pertussis toxin (PTX) and possibly involves G-proteins $G_{i\alpha 2}$ and $G_{i\alpha 3}$ (15, 16). On the other hand, PTX-resistant G-protein α subunits of the G_q class can mediate phospholipid hydrolysis in platelets (17). PAF stimulates phospholipid turnover through phospholipases PLCγ and PLCβ in many systems,

including platelets, macrophages, B cell lines and endothelial cells (2, 18, 19). It also activates PLA_2, PLD and mitogen-activated kinase (MAPK) in many different cells and tissues (2, 20). PAF induces tyrosine phosphorylation of numerous cellular proteins, such as $p125^{fak}$ in human endothelial cells and brain (21, 22), p85 regulatory subunit of phosphatidilinositol 3-kinase (PI3K) (23), $pp60^{src}$ (24), Fyn, Syk and Lyn in a human B cell line (23, 25).

The Janûs kinase/Signal Transducers and Activators of Transcription (Jak/STAT) pathway is recognized as one of the major mechanisms by which cytokine receptors transduce intracellular signals. To date, four mammalian Jaks have been identified (Jak1, Jak2, Jak3 and Tyk2) and seven STAT proteins have been characterized (STAT1-STAT6) (26). Cytokine-induced oligomerization of receptors will activate Jaks, which will then phosphorylate specific tyrosine residues of the receptor, providing binding sites for several SH2-domain-containing proteins, including STATs. These will then bind to the phosphorylated receptor and themselves become targets for Jaks. The phosphorylated STATs will then dimerize and translocate to the nucleus to induce transcription of specific genes (27).

The Jak/STAT signaling pathway is not a unique feature of cytokine receptors. There is now compelling evidence of their importance in GPCR signaling. Marrero and colleagues provided the first evidence that the Jak/STAT pathway can be stimulated by the angiotensin II AT1 receptor (28). Later, activation of the Jak2/STAT3 pathway had been demonstrated for the CCR2B (29) and 5-HT2a receptors (30) and phosphorylation of Jak2 and Jak3 by the chemokine CCR5 (31) and CXCR4 (32) receptors has been shown.

Jak family members are constitutively associated with cytokine receptors and become activated by auto- and/or trans-phosphorylation after cytokine binding to the extracellular domain of its cognate receptor (33). In contrast to cytokine receptors, Jak2 association with GPCRs has been shown to occur only after agonist exposure, except in the case of PAFR, where we have found that Tyk2 is constitutively associated with the receptor (34). Regions of the receptor responsible for Jak2 association have been studied only in a limited number of GPCRs and were shown to be the DRY motif in the second intracellular loop of CCR2B (29) and a proline-rich motif, YIPP, in the C-terminal tail of the angiotensin AT1 receptor (35). However, recent studies indicated Jak2 association with the AT1 receptor was indirect and mediated by SHP-2 acting as a scaffolding protein (36).

Recently, we showed that Tyk2 was essential for PAF-induced PAFR transcriptional activation. PAF induced a rapid, G-protein-independent, activation of Tyk2 in the myeloid cell line MonoMac-1 as well as in a transfected cell system (34). Tyk2 was constitutively associated with the PAFR, analogous to the cytokine receptors. Here we provide evidence that, in addition to Tyk2, PAF induces Jak2 activation in myeloid cells as well as in a reconstituted system. Interestingly, catalytically active Tyk2 is necessary for Jak2 phosphorylation and association with PAFR. In addition, PAF-mediated Jak2 activation is independent of G-proteins and receptor internalization. Finally, the C-terminus of the receptor is important for PAF-stimulated Jak2 activation.

Materials and Methods

Materials

PAF was from the Cayman Chemical Company (Ann Arbor, MI, USA). WEB 2086 was from Boehringer Ingelheim (Laval, QC, Canada). Human Interferon-γ was acquiered from PeproTech Canada Inc. (Ottawa, Ont, Canada). FuGENE^{TM}6 Transfection Reagent was from Roche Molecular Biochemicals (Laval, QC, Canada). Antibodies used were rabbit polyclonal anti-Jak2, monoclonal anti-phosphotyrosine PY20 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); monoclonal anti-c-Myc was from ATCC (Manassas, VA, USA). Horseradish peroxidase-conjugated goat anti-mouse and donkey anti-rabbit antibodies were from Amersham Pharmacia Biotech. (Baie d'Urfé, Qc, Canada). Human Tyk2 WT and mutant cDNAs were a kind gift of Dr. J. Krolewski (University of California, CA, USA). Rat Jak2 cDNA was produced as described (37). Clone Kp132 containing the hPAFR cDNA was kindly provided by Dr. R. Ye (The Scripps Research Institute, La Jolla, CA, USA). MonoMac-1 (DSM ACC252) cells were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Dr. G. Guillemette (Université de Sherbrooke, Canada) kindly provided COS-7 cells.

Cell Culture and Transfection.

COS-7 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Burlington, On, Canada) supplemented with 10% fetal bovine serum (Sigma, Oakville, On, Canada) and 100 μg/ml gentamicin. Cells were plated in 100 mm dishes ($1x10^6$ cells/dish) (Sarstedt, Montréal, Qc, Canada) and transiently transfected with human

PAFR cDNA cloned into the pcDNA3 expression vector with or without, rJak2 or pcDNA3 cDNA (6 μg of DNA, total) using 12 μl of FuGENE. 48 h after transfection, cells were incubated without serum for 1-3 h, then left unstimulated or stimulated with PAF (10^{-7}M). Cells were lysed and extracts used as indicated below for immunoprecipitation.

MonoMac-1 cells were maintained in RPMI medium (Invitrogen) supplemented with 10 mM HEPES, 10 % fetal bovine serum, 100 U/ml ampicillin, 100 μg/ml streptomycin, 10 mM nonessential amino acids and 10 mM sodium pyruvate.

Construction of Mutant PAFR.

To construct truncated mutant V330Stop, the polymerase chain reaction product generated with the oligonucleotide 5'CGGGATCCTAAACCACTTCAGTGACCGT-3' and the M13 sequencing primer 5'-GTAAAACGACGGCCAGT-3'using Kp132 as template was subcloned into the *c-myc*-tagged receptor coding sequence using BstEII and BamH1 restriction enzymes. Mutation was confirmed by sequencing (University of Calgary, Alberta, Canada). Mutants K298Stop, C317Stop, D289A, T305Stop, M311Stop and minigene encoding 46 C-terminal amino acids of PAFR were described previously (38-40).

Immunoprecipitation and Western Blotting.

MonoMac-1 cells ($20x10^6$) were incubated in medium without serum for 24 h, stimulated with PAF (10^{-8}M) for indicated times and lysed in buffer: 50 mM Tris pH 7.5, 1 mM EGTA, 150 mM NaCl, 1 mM NaF, 1 mM Na_3VO_4, 1% NP-40, 1 mM PMSF,

0.25% sodium deoxycholate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin (Sigma), 30 min on ice. Lysates were incubated with anti-Jak2 or anti-Myc Abs overnight at 4^0C. Proteins of interest were then immunoprecipitated by incubation with 100 μg of Protein A-Sepharose, 2 h at 4^0C. After washing 4 times in 0.5X lysis buffer, complexes were dissociated in loading buffer (300mM Tris pH 6.8, 24% glycerol, 5% SDS, 143 mM 2-mercaptothanol); separated on 10% SDS-PAGE and transferred to PVDF membranes. Non-specific sites on membranes were blocked with Tris-buffered saline (TBS) with 2.5% gelatin for 1 h and incubated with anti-Tyr(P) in TBS-Tween 0.1% + 0.5% gelatin overnight at 4^0C. After washing and incubation with secondary antibodies, an enhanced chemiluminescent detection system was used for protein detection (Amersham). Membranes were stripped by incubation in 62.5 mM Tris-HCl pH 6.8, 2% SDS and 10 mM 2-ME for 30 min at 50^0C. After washing, membranes were reprobed with appropriate antibodies, and developed as described above. To confirm receptor specificity, cells were pretreated with WEB 2086 (10^{-6}M) 20 min at 37^0C before PAF stimulation.

Results

In the present study, we investigated the mechanism of Jak2 activation in response to PAF. Initially, we pretreated, or not, MonoMac-1 cells for 20 min with the PAF specific antagonist, WEB2086 (10^{-6}M), then the cells were stimulated by PAF (10^{-9}M) for 1 min. Cell lysates were immunoprecipitated with anti-phosphotyrosine antibody, resolved by SDS-PAGE and immunoblotted with anti-Jak2 antibodies. As illustrated in Fig. 1A, PAF induced a rapid Jak2 tyrosine phosphorylation, which was completely blocked by WEB2086, indicating a receptor-mediated event. In kinetic studies (Fig. 1B), we observed a rapid increase in Jak2 phosphorylation in cells treated with PAF, which was sustained for 20 min and declined thereafter. In parallel, IFN-γ (200U/ml) was used as positive control, as it is known to activate Jak1 and Jak2 (Fig. 1B). Similar results were obtained when the order of antibody addition was reversed, and anti-Jak2 immunoprecipitates were immunoblotted with anti-phosphotyrosine (Figure 1C).

Subsequently, in order to find a system where we could dissect the mechanism(s) of Jak2 activation more easily, we used COS-7 cells transfected with PAFR and Jak2 cDNAs. Forty-eight hours after transfection, quiescent COS-7 cells were treated with PAF for indicated times and lysed. The cell lysates were subjected to immunoprecipitation with anti-Jak2 antibodies and immunoblotted with anti-phosphotyrosine antibodies. As shown in Figure 2, PAF induced a rapid 3.8-fold increase in Jak2 tyrosine phosphorylation which was comparable to that observed in MonoMac-1 cells.

Previously, we had found that Tyk2 association with PAFR was independent of agonist binding and G-protein coupling (34). In order to examine whether Jak2 could associate with PAFR, lysates from COS-7 cells transfected with Jak2 and cMyc-tagged PAFR cDNAs were immunoprecipitated with anti-Myc antibodies and analyzed by Western blot with anti-Jak2 antibodies. No co-immunoprecipitation between the receptor and kinase was observed either in PAF-stimulated or unstimulated cells (data not shown). Under the same conditions, but with additional co-transfection of Tyk2 cDNA, ligand-dependent Jak2 association with the receptor was detected (Fig. 3A). Moreover, as shown in Fig. 3B (middle), the kinase inactive Tyk2 mutant, K930I, was also constitutively associated with PAFR, but Jak2 was not immunoprecipitated in these conditions (Fig. 3B, top), suggesting that the presence of active Tyk2 was important for Jak2 association with the receptor. Finally, the membranes were re-blotted with anti-Myc antibodies to show that equivalent amounts of PAFR were immunoprecipitated (bottom panels). Interestingly, in contrast to Tyk2, Jak2 association with the receptor was ligand-dependent and transient.

Subsequently, we were interested to determine whether Tyk2 kinase activity was involved in PAF-induced Jak2 activation or whether Tyk2 served simply a scaffolding function. We used COS-7 cells transfected with cDNAs of PAFR, Jak2 and WT Tyk2 or the catalytically inactive Tyk2 mutant, K930I. Jak2 tyrosine phosphorylation was assessed before and after 1 min of PAF stimulation. Figure 4 shows that PAF-induced Jak2 tyrosine phosphorylation was completely inhibited by co-transfection of the dominant-negative Tyk2:K930I mutant. Tyk2:K930I, itself, was not phosphorylated in

response to ligand (results not illustrated). These results suggest that Tyk2 could activate Jak2 in response to PAF via transphosphorylation.

Using a mutant PAFR receptor, our results indicated that similarly to Tyk2, Jak2 activation by PAF was independent of G-protein coupling. As illustrated in Fig. 5A, when COS-7 cells were transfected with the G-protein-uncoupled mutant PAFR:D289A and Jak2 cDNAs, PAF induced a rapid Jak2 tyrosine phosphorylation. But despite a 4-fold higher affinity for ligand than the WT receptor (34, 39), this mutant demonstrated only a 2.3-2.6-fold increase in Jak2 phosphorylation after 1 min of PAF treatment. In addition, kinase phosphorylation induced by PAFR:D289A was more transient when compared with WT PAFR (Fig. 5B). Our group showed, recently, that the PAFR:D289A mutant failed to induce arrestin translocation and to internalize (40), suggesting that Jak2 activation was also independent of receptor internalization.

The DRY and YIPP motifs identified as Jak2 binding regions in other GPCRs (29, 35) are not found in the PAFR. However, there is a tyrosine present in the C-tail of the receptor and we were therefore interested in determining whether this part of the receptor was important for PAF-mediated Jak2 activation. Initially, we used a construct encoding the 46 C-terminal amino acids of PAFR, in addition to PAFR and Jak2 cDNAs, in transiently transfected COS-7 cells. As illustrated in Fig. 6A, co-transfection of this minigene construct partially inhibited PAF-mediated Jak2 activation. In addition, we investigated ligand-induced Jak2 phosphorylation using PAFR C-terminus deletion mutants. As determined by densitometric analysis, deletion of the last twelve C-terminal residues in the V330Stop mutant resulted in a 20% decrease in Jak2 phosphorylation compared to WT PAFR. Removal of the next 8 to 25 residues (the PAFR mutants

C317Stop, M311Stop, and T305Stop), further decreased, but did not eliminate Jak2 activation (Fig. 6B). With the exception of the T305Stop mutant, all the mutant receptors have ligand affinity and cell surface expression similar to WT receptor (24, 34, 35). Interestingly, despite the truncation of twelve residues from 317 to 305, including the tyrosine309, the mutant receptors were able to maintain a comparable degree of Jak2 phosphorylation. Finally, PAFR:K298Stop, the mutant receptor which is missing the entire C-tail, failed to significantly activate Jak2 in response to PAF. These data suggest that PAFR has at least two domains in its C-terminus important for Jak2 activation, one in close proximity to the membrane (a.a. 298-305) and another, more distant (a.a. 317-330).

Discussion

The Jak/STAT pathway has been shown to participate in the signaling initiated not only by cytokines and growth factors, but also by GPCRs. Whereas several GPCRs activate the Jak/STAT pathway, the mechanism(s) of this activation are still poorly defined. Results presented in this report indicate that the PAF receptor can activate Jak2 via a G-protein- and internalization-independent mechanism. Jak2 association with the PAFR is ligand-dependent and requires the presence of a catalytically active Tyk2. Moreover, the C-terminus of the PAF receptor contains important domains for Jak2 activation.

We showed that PAF induces activation of Jak2 in a myeloid cell line MonoMac-1 as well as in transfected COS-7 cells. This activation was receptor-mediated, as the PAF antagonist WEB2086 inhibited PAF-induced Jak2 phosphorylation.

The kinetics of PAF-stimulated Jak2 activation were similar to those observed after angiotensin II (28) and thrombin (41) stimulation of rat aortic smooth muscle (RASM) cells. Comparable activation times had also been observed for various cytokine receptors, in particular, Jak2 induction by IL-12 in T cells (42), and Jak1 and Jak2 phosphorylation in RASM cells upon IFN-α and IFN-γ stimulation, respectively (28). In contrast, Jak2 activation was more transient for the chemokine CCR5 and CXCR4 receptors (31, 32). Previously, we had found that PAF also induced a rapid Tyk2 activation in MonoMac-1 cells that declined to basal levels after 5 min of PAF treatment (34). More prolonged Jak2 activation had been demonstrated in rat fetal myoblasts in response to serotonin (30) and in Ba/F3 pro-B-lymphocyte cells after α-MSH binding to the melanocortin MC5 (43) receptors. Recently, Deo and colleagues demonstrated that

PAFR antagonists inhibited basic Fibroblast Growth Factor (bFGF)-simulated Jak2 phosphorylation in HUVEC cells suggesting the involvement of the PAFR pathway in the bFGF-mediated Jak2 activation (44). Under these conditions, Jak2 phosphorylation was maintained for longer periods of time, which may indicate either cell-specific differences in PAF-induced stimulation, or the temporal modulation of the bFGF and PAFR signaling pathways in Jak2 phosphorylation.

GPCRs have also been shown to induce Jak2 activation in a reconstituted system. For example, Jak2 tyrosine phosphorylation in response to angiotensin II was observed in COS-7 cells transfected with angiotensin AT1 receptor and Jak2 cDNAs (45). We used this system, given that myeloid cells are notoriously difficult to transfect, to further dissect the mechanism of Jak2 activation, using mutant forms of PAFR and Tyk2.

We have also found that Jak2, unlike Tyk2 (34), was not constitutively associated with the PAF receptor. However, Jak2 could be co-immunoprecipitated with the receptor after PAF stimulation, but only in the presence of catalytically active Tyk2. The association of Jak2 with other GPCRs had also been shown to be ligand-dependent (28, 29, 32), unlike the stable association of Janus family members with cytokine receptors. Jaks are known to function as scaffolding proteins, interacting with receptors and STATs (46, 47). At this point, we can only speculate that phosphorylated Tyk2 would serve as a docking site for Jak2. It is unlikely that it is the receptor which is phosphorylated by Tyk2, since removal of the only tyrosine residue in the C-terminus of PAFR still permits Jak2 activation. It would therefore be interesting to investigate whether PAF stimulation induces a direct association between the two kinases. Alternately, other proteins could serve this function; PAF stimulates cSrc phosphorylation (24) and it had been shown that

angiotensin II-induced association of Jak2 and c-Src requires the N-terminus of Jak2 and the SH2 domain of c-Src kinase (45). Although the functional significance of this interaction was not clarified, it has been proposed that Jak2 and Src kinases could act synergistically on a given substrate.

After activation, PAFR recruits members of the arrestin family which are capable of both propagating and terminating signals from GPCRs. Arrestins have been shown to act as scaffolding proteins (48) and they have been shown to recruit Src kinases as well as MAP kinases to the receptor complex (49, 50). In our hands, the PAFR mutants T305Stop, M311Stop, C317Stop and D289A which do not induce arrestin translocation, nevertheless stimulated Jak2 phosphorylation. These results suggest that PAF-mediated Jak2 activation is independent of arrestins.

It is generally assumed that upon receptor stimulation, Jaks become activated via a process of transphosphorylation. Experiments showing a kinase hierarchy in Jak hetero-oligomers provide the strongest support for a trans-phosphorylation mechanism (33). A dominant-negative Jak1 prevents the activation of Tyk2 after IFN-α stimulation in 293T cells (51), and inactive Jak2 blocks the activation of Jak1 in response to IFN-γ receptor stimulation (52). We observed inhibition of PAF-mediated Jak2 activation by co-transfection of a dominant-negative Tyk2:K930I mutant. Our data are consistent with the general paradigm of Jak trans-phosphorylation, as seen in response to cytokine stimulation. It is presumed that Jaks initially have a low basal activity, which is increased upon activation-loop phosphorylation (33). Several groups have shown the importance of trans-phosphorylation on tyrosine residues in the enzyme activation loop. Gauzzi and colleagues demonstrated that Tyk2 activation is regulated by phosphorylation

of tyrosines Tyr-1054 and Tyr-1055 in the activation loop, and this event is dependent on the induced activity of Jak1 in response to IFN-α in the Tyk2 deficient 11.1 cell line (53). On the other hand, kinase basal activity depends on the enzyme autophosphorylation state, and mutation of critical tyrosine residues to phenylalanine results in a kinase unresponsive to receptor stimulation. Recent studies demonstrated that mutation of Tyr-1007 in Jak2 kinase prevented Jak2 kinase activity. Moreover, in γ2 human fibrosarcoma cells which lack Jak2, the Tyr1007Phe mutant of Jak2 failed to respond to Epo stimulation, suggesting that Jak2 activation is dependent on Jak2 itself (54). Results of Zhou and colleagues indicated that Tyr980Phe mutant of Jak3 positively regulates its kinase activity whereas Tyr981 has a negative regulatory function (55). With the exception of CCR2B, which seems to signal only through Jak2 (29), all the other GPCR are known to induce activation of several Jaks. However, kinase hierarchy, if any exists, has not yet been studied. To further clarify Jak2-Tyk2 kinase interdependence in PAF-mediated signaling, it will be interesting to use Jak kinase deficient cell lines as well as kinase mutants with specific mutated tyrosine residues.

Using a co-transfection system, we showed that the G-protein-uncoupled PAFR:D289A mutant could induce Jak2 phosphorylation, indicating that PAFR induces Jak2 phosphorylation independently of G-protein activation. Activation of the Jak2/STAT3 pathway by CCR2B and CXCR4 was not inhibited by pertussis toxin, suggesting a $G_{\alpha i}$-independent action. (29, 32). The action of other G-proteins could not be ruled out in these studies. The use of the PAFR:D289A mutant which does not activate either $G_{\alpha i}$ or $G_{\alpha q}$ proteins, strongly indicates that activation of the Jak/STAT pathway by GPCRs can be truly G-protein-independent.

Very little is known about the receptor regions involved in GPCR-Jak interactions. Ali and colleagues demonstrated that the C-terminus of the rat AT1 receptor binds Jak2, and this association is dependent on the receptor motif YIPP (35). This motif also appeared to be important in angiotensin II-dependent tyrosine phosphorylation of Jak2. Previously, a similar motif, YIIP found within the platelet-derived growth factor receptor and the motif YLPP, within the epidermal growth factor receptor, have been shown to be SH2 target sequences (56). While originally Jak2 interaction with the AT1 receptor was thought to be direct, subsequent studies revealed that antibodies against SHP-2 phosphatase could block Jak2/AT1 receptor interaction. SHP-2 binds to the same YIPP motif in the C-terminus of the AT1 receptor that was required for Jak2 association and appears to serve as an adaptor protein for Jak2 (36). The substitution mutant Tyr139Phe of CCR2B, when expressed in HEK293 cells, did not trigger Jak2/STAT3 activation and $G_{\alpha i}$ association to the receptor, suggesting the involvement of the tyrosine within the conserved DRY motif, within the second intracellular loop of the receptor, both in G-protein and Jak2 activation (29). Altogether, these results suggest that different receptor motifs and possibly adaptor molecules are important in the modulation of Jak activity and in the regulation of GPCR-Jak interaction. In the PAFR, the residues corresponding to the DRY motif are NRF, lacking the conserved tyrosine residue in the second intracellular loop. A tyrosine is found in the C-tail of PAFR and we therefore investigated the role of this region for the activation of Jak2. Co-transfection of a minigene, bearing the C-terminus of the receptor, resulted in significantly diminished kinase activation. We then used serial deletion mutants to determine which portion of the C-terminus was involved in PAFR-Jak2 interaction. The receptor C-terminal deletion

mutants were still able, although less effectively than WT, to activate Jak2. This included the mutants in which tyrosine309 was eliminated, indicating that this residue was not essential for Jak2 activation. Only a mutant which is lacking the entire C-terminus failed to activate Jak2. These results indicate that the conformation of the C-terminus is possibly important in Jak2 activation, either by direct interaction, after activation of the receptor, or via another protein, possibly Tyk2.

In summary, this report provides evidence, for the first time, of a hierarchical activation of members of the Jak family by a GPCR. In addition, it defines the mechanism of Jak2 activation as G-protein- and arrestin-independent and identifies the PAFR C-terminus as the region of the receptor essential for Jak2 activation.

References

1. Braquet, P., L. Touqui, T. Y. Shen, and B. B. Vargaftig. 1987. Perspectives in platelet-activating factor research. *Pharmacol Rev 39:97.*

2. Izumi, T., and T. Shimizu. 1995. Platelet-activating factor receptor: gene expression and signal transduction. *Biochim Biophys Acta 1259:317.*

3. Braquet, P., and M. Rola-Pleszczynski. 1987. The role of PAF in immunological responses: a review. *Prostaglandins 34:143.*

4. Peplow, P. V. 1999. Regulation of platelet-activating factor (PAF) activity in human diseases by phospholipase A2 inhibitors, PAF acetylhydrolases, PAF receptor antagonists and free radical scavengers. *Prostaglandins Leukot Essent Fatty Acids 61:65.*

5. Cuss, F. M., C. M. Dixon, and P. J. Barnes. 1986. Effects of inhaled platelet activating factor on pulmonary function and bronchial responsiveness in man. *Lancet 2:189.*

6. Archer, C. B., C. P. Page, J. Morley, and D. M. MacDonald. 1985. Accumulation of inflammatory cells in response to intracutaneous platelet activating factor (Paf-acether) in man. *Br J Dermatol 112:285.*

7. Dubois, C., E. Bissonnette, and M. Rola-Pleszczynski. 1989. Platelet-activating factor (PAF) enhances tumor necrosis factor production by alveolar macrophages. Prevention by PAF receptor antagonists and lipoxygenase inhibitors. *J Immunol 143:964.*

8. Denault, S., M. J. April, and J. Stankova. 1997. Transcriptional activation of the interleukin-8 gene by platelet- activating factor in human peripheral blood monocytes. *Immunology 91:297.*

9. Pignol, B., S. Henane, B. Sorlin, M. Rola-Pleszczynski, J. M. Mencia-Huerta, and P. Braquet. 1990. Effect of long-term treatment with platelet-activating factor on IL-1 and IL-2 production by rat spleen cells. *J Immunol 145:980.*

10. Poubellê, P. E., D. Gingras, C. Demers, C. Dubois, D. Harbour, J. Grassi, and M. Rola-Pleszczynski. 1991. Platelet-activating factor (PAF-acether) enhances the concomitant production of tumour necrosis factor-alpha and interleukin-1 by subsets of human monocytes. *Immunology 72:181.*

11. Rola-Pleszczynski, M., and J. Stankova. 1992. Differentiation-dependent modulation of TNF production by PAF in human HL-60 myeloid leukemia cells. *J Leukoc Biol 51:609.*

12. Thivierge, M., and M. Rola-Pleszczynski. 1992. Platelet-activating factor enhances interleukin-6 production by alveolar macrophages. *J Allergy Clin Immunol 90:796.*

13. Vercellotti, G. M., H. Q. Yin, K. S. Gustafson, R. D. Nelson, and H. S. Jacob. 1988. Platelet-activating factor primes neutrophil responses to agonists: role in promoting neutrophil-mediated endothelial damage. *Blood 71:1100.*

14. Rola-Pleszczynski, M. 1990. Priming of human monocytes with PAF augments their production of tumor necrosis factor. *J Lipid Mediat 2:S77.*

15. Huang, S. J., P. N. Monk, C. P. Downes, and A. D. Whetton. 1988. Platelet-activating factor-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate

stimulates the production of reactive oxygen intermediates in macrophages. *Biochem J 249:839.*

16. Naccache, P. H., M. M. Molski, M. Volpi, E. L. Becker, and R. I. Sha'afi. 1985. Unique inhibitory profile of platelet activating factor induced calcium mobilization, polyphosphoinositide turnover and granule enzyme secretion in rabbit neutrophils towards pertussis toxin and phorbol ester. *Biochem Biophys Res Commun 130:677.*

17. Dhar, A., A. K. Paul, and S. D. Shukla. 1990. Platelet-activating factor stimulation of tyrosine kinase and its relationship to phospholipase C in rabbit platelets: studies with genistein and monoclonal antibody to phosphotyrosine. *Mol Pharmacol 37:519.*

18. Chao, W., and M. S. Olson. 1993. Platelet-activating factor: receptors and signal transduction. *Biochem J 292:617.*

19. Shukla, S. D. 1991. Inositol phospholipid turnover in PAF transmembrane signalling. *Lipids 26:1028.*

20. Liu, B., S. Nakashima, H. Kanoh, T. Takano, T. Shimizu, and Y. Nozawa. 1994. Activation of phospholipase D in Chinese hamster ovary cells expressing platelet-activating factor receptor. *J Biochem (Tokyo) 116:882.*

21. Soldi, R., F. Sanavio, M. Aglietta, L. Primo, P. Defilippi, P. C. Marchisio, and F. Bussolino. 1996. Platelet-activating factor (PAF) induces the early tyrosine phosphorylation of focal adhesion kinase (p125FAK) in human endothelial cells. *Oncogene 13:515.*

22. Calcerrada, M. C., R. E. Catalan, M. J. Perez-Alvarez, B. G. Miguel, and A. M. Martinez. 1999. Platelet-activating factor stimulation of p125(FAK) and p130(Cas) tyrosine phosphorylation in brain. *Brain Res 835:275.*

23. Kuruvilla, A., C. Pielop, and W. T. Shearer. 1994. Platelet-activating factor induces the tyrosine phosphorylation and activation of phospholipase C-gamma 1, Fyn and Lyn kinases, and phosphatidylinositol 3-kinase in a human B cell line. *J Immunol 153:5433.*

24. Dhar, A., and S. D. Shukla. 1994. Electrotransjection of pp60v-src monoclonal antibody inhibits activation of phospholipase C in platelets. A new mechanism for platelet-activating factor responses. *J Biol Chem 269:9123.*

25. Rezaul, K., K. Sada, T. Inazu, and H. Yamamura. 1997. Platelet-activating factor stimulates calcium-dependent activation of protein-tyrosine kinase Syk in a human B cell line. *Biochem Biophys Res Commun 239:23.*

26. Liu, K. D., S. L. Gaffen, and M. A. Goldsmith. 1998. JAK/STAT signaling by cytokine receptors. *Curr Opin Immunol 10:271.*

27. Watanabe, S., and K. Arai. 1996. Roles of the JAK-STAT system in signal transduction via cytokine receptors. *Curr Opin Genet Dev 6:587.*

28. Marrero, M. B., B. Schieffer, W. G. Paxton, L. Heerdt, B. C. Berk, P. Delafontaine, and K. E. Bernstein. 1995. Direct stimulation of Jak/STAT pathway by the angiotensin II AT1 receptor. *Nature 375:247.*

29. Mellado, M., J. M. Rodriguez-Frade, A. Aragay, G. del Real, A. M. Martin, A. J. Vila-Coro, A. Serrano, F. Mayor, Jr., and A. C. Martinez. 1998. The chemokine

monocyte chemotactic protein 1 triggers Janus kinase 2 activation and tyrosine phosphorylation of the CCR2B receptor. *J Immunol 161:805.*

30. Guillet-Deniau, I., A. F. Burnol, and J. Girard. 1997. Identification and localization of a skeletal muscle serotonin 5-HT2A receptor coupled to the Jak/STAT pathway. *J Biol Chem 272:14825.*

31. Wong, M., S. Uddin, B. Majchrzak, T. Huynh, A. E. Proudfoot, L. C. Platanias, and E. N. Fish. 2001. Rantes activates Jak2 and Jak3 to regulate engagement of multiple signaling pathways in T cells. *J Biol Chem 276:11427.*

32. Vila-Coro, A. J., J. M. Rodriguez-Frade, A. Martin De Ana, M. C. Moreno-Ortiz, A. C. Martinez, and M. Mellado. 1999. The chemokine SDF-1alpha triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. *Faseb J 13:1699.*

33. Duhe, R. J., L. H. Wang, and W. L. Farrar. 2001. Negative regulation of Janus kinases. *Cell Biochem Biophys 34:17.*

34. Lukashova, V., C. Asselin, J. J. Krolewski, M. Rola-Pleszczynski, and J. Stankova. 2001. G-protein-independent activation of Tyk2 by the platelet-activating factor receptor. *J Biol Chem 276:24113.*

35. Ali, M. S., P. P. Sayeski, L. B. Dirksen, D. J. Hayzer, M. B. Marrero, and K. E. Bernstein. 1997. Dependence on the motif YIPP for the physical association of Jak2 kinase with the intracellular carboxyl tail of the angiotensin II AT1 receptor. *J Biol Chem 272:23382.*

36. Marrero, M. B., V. J. Venema, H. Ju, D. C. Eaton, and R. C. Venema. 1998. Regulation of angiotensin II-induced JAK2 tyrosine phosphorylation: roles of SHP-1 and SHP-2. *Am J Physiol 275:C1216.*

37. Smith, M. R., R. J. Duhe, Y. Liu, and W. L. Farrar. 1997. Microinjected cDNA encoding JAK2 protein-tyrosine kinase induces DNA synthesis in NIH 3T3 cells. *FEBS Lett 408:327.*

38. Le Gouill, C., J. L. Parent, M. Rola-Pleszczynski, and J. Stankova. 1997. Structural and functional requirements for agonist-induced internalization of the human platelet-activating factor receptor. *J Biol Chem 272:21289.*

39. Parent, J. L., C. L. Gouill, E. Escher, M. Rola-Pleszczynski, and J. Stankova. 1996. Identification of transmembrane domain residues determinant in the structure-function relationship of the human platelet-activating factor receptor by site-directed mutagenesis. *J Biol Chem 271:23298.*

40. Chen, Z., D. J. Dupre, C. Le Gouill, M. Rola-Pleszczynski, and J. Stankova. 2002. Agonist-induced internalization of the platelet-activating factor receptor is dependent on arrestins but independent of G-protein activation. Role of the C terminus and the (D/N)PXXY motif. *J Biol Chem 277:7356.*

41. Madamanchi, N. R., S. Li, C. Patterson, and M. S. Runge. 2001. Thrombin regulates vascular smooth muscle cell growth and heat shock proteins via the JAK-STAT pathway. *J Biol Chem 276:18915.*

42. Bacon, C. M., D. W. McVicar, J. R. Ortaldo, R. C. Rees, J. J. O'Shea, and J. A. Johnston. 1995. Interleukin 12 (IL-12) induces tyrosine phosphorylation of JAK2

and TYK2: differential use of Janus family tyrosine kinases by IL-2 and IL- 12. *J Exp Med 181:399.*

43. Buggy, J. J. 1998. Binding of alpha-melanocyte-stimulating hormone to its G-protein- coupled receptor on B-lymphocytes activates the Jak/STAT pathway. *Biochem J 331:211.*

44. Deo, D. D., T. W. Axelrad, E. G. Robert, V. Marcheselli, N. G. Bazan, and J. D. Hunt. 2002. Phosphorylation of STAT-3 in response to basic fibroblast growth factor occurs through a mechanism involving platelet-activating factor, JAK-2, and Src in human umbilical vein endothelial cells. Evidence for a dual kinase mechanism. *J Biol Chem 277:21237.*

45. Sayeski, P. P., M. S. Ali, K. Hawks, S. J. Frank, and K. E. Bernstein. 1999. The angiotensin II-dependent association of Jak2 and c-Src requires the N-terminus of Jak2 and the SH2 domain of c-Src. *Circ Res 84:1332.*

46. Fujitani, Y., M. Hibi, T. Fukada, M. Takahashi-Tezuka, H. Yoshida, T. Yamaguchi, K. Sugiyama, Y. Yamanaka, K. Nakajima, and T. Hirano. 1997. An alternative pathway for STAT activation that is mediated by the direct interaction between JAK and STAT. *Oncogene 14:751.*

47. Ali, M. S., P. P. Sayeski, and K. E. Bernstein. 2000. Jak2 acts as both a STAT1 kinase and as a molecular bridge linking STAT1 to the angiotensin II AT1 receptor. *J Biol Chem 275:15586.*

48. Milano, S. K., H. C. Pace, Y. M. Kim, C. Brenner, and J. L. Benovic. 2002. Scaffolding functions of arrestin-2 revealed by crystal structure and mutagenesis. *Biochemistry 41:3321.*

49. Luttrell, L. M., S. S. Ferguson, Y. Daaka, W. E. Miller, S. Maudsley, G. J. Della Rocca, F. Lin, H. Kawakatsu, K. Owada, D. K. Luttrell, M. G. Caron, and R. J. Lefkowitz. 1999. Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science 283:655.*

50. Luttrell, L. M., F. L. Roudabush, E. W. Choy, W. E. Miller, M. E. Field, K. L. Pierce, and R. J. Lefkowitz. 2001. Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. *Proc Natl Acad Sci U S A 98:2449.*

51. Krishnan, K., R. Pine, and J. J. Krolewski. 1997. Kinase-deficient forms of Jak1 and Tyk2 inhibit interferon alpha signaling in a dominant manner. *Eur J Biochem 247:298.*

52. Briscoe, J., N. C. Rogers, B. A. Witthuhn, D. Watling, A. G. Harpur, A. F. Wilks, G. R. Stark, J. N. Ihle, and I. M. Kerr. 1996. Kinase-negative mutants of JAK1 can sustain interferon-gamma-inducible gene expression but not an antiviral state. *Embo J 15:799.*

53. Gauzzi, M. C., L. Velazquez, R. McKendry, K. E. Mogensen, M. Fellous, and S. Pellegrini. 1996. Interferon-alpha-dependent activation of Tyk2 requires phosphorylation of positive regulatory tyrosines by another kinase. *J Biol Chem 271:20494.*

54. Feng, J., B. A. Witthuhn, T. Matsuda, F. Kohlhuber, I. M. Kerr, and J. N. Ihle. 1997. Activation of Jak2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop. *Mol Cell Biol 17:2497.*

55. Zhou, Y. J., E. P. Hanson, Y. Q. Chen, K. Magnuson, M. Chen, P. G. Swann, R. L. Wange, P. S. Changelian, and J. J. O'Shea. 1997. Distinct tyrosine

phosphorylation sites in JAK3 kinase domain positively and negatively regulate its enzymatic activity. *Proc Natl Acad Sci U S A 94:13850.*

56. Pascal, S. M., A. U. Singer, G. Gish, T. Yamazaki, S. E. Shoelson, T. Pawson, L. E. Kay, and J. D. Forman-Kay. 1994. Nuclear magnetic resonance structure of an SH2 domain of phospholipase C-gamma 1 complexed with a high affinity binding peptide. *Cell 77:461.*

ACKNOWLEDGMENTS

The authors would like to acknowledge the excellent technical advice and collaboration of Denis Gingras and Sylvie Turcotte. We also thank Dr. J. Krolewski for generously providing hTyk2 cDNA clones and Dr. C. Le Gouill for a critical review of the manuscript.

The abbreviations used are: G-protein, GTP-binding regulatory protein; GPCR, G-protein-coupled receptor; Jak, Janus kinase; PTX, pertussis toxin; PAF, platelet-activating factor; PAFR, platelet-activating factor receptor

FIGURE LEGENDS

Figure 1. Specificity of PAF-induced Jak2 activation.

A. MonoMac-1 cells were unstimulated (NS) or stimulated with PAF (10^{-9}M) for 1 min.; WEB2086 (10^{-6}M), a PAF receptor antagonist, was added 20 min before PAF. Cell lysates were immunoprecipitated (IP) with anti-phosphotyrosine antibodies. Immune complexes, fractionated by SDS-PAGE, were transferred to a PVDF membrane, which was revealed (blot) with anti-Jak2 antibodies.

B. MonoMac-1 cells were stimulated with PAF for indicated times and with IFN-γ (200U/ml) for 10 min. Jak2 tyrosine phosphorylation was assessed by immunoprecipitation with anti-phosphotyrosine (pTyr) antibodies and Western blotting with anti-Jak2 antibodies. Results shown are representative of 3 independent experiments.

C. MonoMac-1 cells were stimulated with PAF for indicated times. Jak2 tyrosine phosphorylation was assessed by immunoprecipitation with anti-Jak2 antibodies and Western blotting with anti-phosphotyrosine (pTyr) antibodies.

Figure 2. Jak2 tyrosine phosphorylation in transfected cells.

A. COS-7 cells were transiently transfected with PAFR and Jak2 cDNAs. 48 hours after transfection, cells were stimulated with PAF for the indicated times. Jak2 tyrosine phosphorylation was assessed by immunoprecipitation with anti-Jak2 antibodies and Western blotting with anti-phosphotyrosine (pTyr) antibodies.

B. Densitometry ratios of pJak2/Jak2 are shown as fold activation, the ratio for unstimulated cells (NS) being set at 1. Results shown are representative of 3 independent experiments.

Figure 3. Jak2 association with PAFR: role of Tyk2.

COS-7 cells were transfected with cmyc-tagged PAFR and Jak2 cDNAs. In addition to PAFR and Jak2, Tyk2 **(A)** or Tyk2 dominant negative mutant K930I **(B)** were co-transfected. 48 hours after transfection, cells were stimulated with PAF for the indicated times. Lysates were immunoprecipitated with anti-Myc Abs and analyzed in Western blot with anti-Jak2 Abs (top). Reprobing of the membrane with anti-Tyk2 Abs assessed Tyk2 or K930I association with PAFR. As a control for the presence of equal amounts of PAFR, the blot was stripped and reblotted with anti-Myc Abs. Results shown are representative of 3 independent experiments.

Figure 4. Dominant-negative Tyk2 blocked PAF-induced Jak2 activation.

COS-7 cells were transiently transfected with PAFR, Jak2 and Tyk2 or dominant-negative Tyk2 mutant, K930I cDNAs. Jak2 tyrosine phosphorylation after 1 min of PAF stimulation was analyzed by immunoprecipitation and Western blotting as in Fig. 2. Results shown are representative of 3 independent experiments.

Figure 5. Jak2 activation by the PAFR G-protein-uncoupled mutant D289A.

A. COS-7 cells were transiently transfected with the PAFR G-protein-uncoupled mutant D289A and Jak2 cDNAs. Jak2 activation was assessed by immunoprecipitation and Western blotting as in Fig. 2. A representative experiment is illustrated.

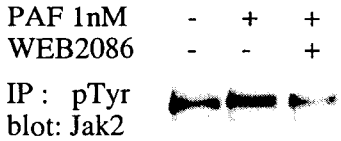
B. Densitometry ratios of pJak2/Jak2 are shown as fold activation, the ratio for unstimulated cells being set at 1. Results shown are mean± S.E of 3 independent experiments.

Figure 6. Inhibition of Jak2 activation by constructs encoding the C-tail of PAFR and by the PAFR C-terminal deletion mutants.

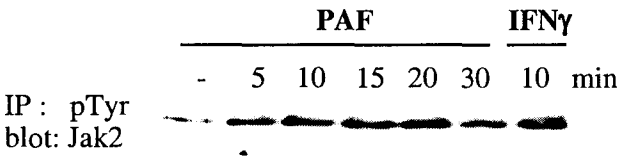
A. COS-7 cells were transiently transfected with PAFR, Jak2 and pcDNA3 or a construct encoding the 46 C-terminal amino acids of PAFR (C-tail) cDNAs. Jak2 tyrosine phosphorylation was analyzed after 1 min of PAF stimulation. The cell lysates were immunoprecipitated with anti-Jak2 antibodies and blotted with anti-phosphotyrosine antibodies. The membrane was then stripped and reblotted with anti-Jak2 antibodies. Results shown are representative of 3 independent experiments.

B. COS-7 cells were transfected with Jak2 and WT PAFR, or WT PAFR + C-tail, or PAFR deletion mutants V330Stop, C317Stop, M311Stop, T305Stop, K298Stop cDNAs. Jak2-immunoprecipitated complexes were probed with anti-phosphotyrosine Abs. Result represent mean± S.E. fold of Jak2 activation after 1 min of PAF treatment (n=4).

A


PAF 1nM - + +
WEB2086 - - +
IP : pTyr
blot: Jak2

B


PAF
IFNγ
- 5 10 15 20 30 10 min
IP : pTyr
blot: Jak2

C

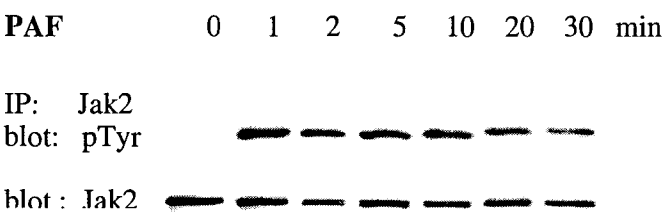
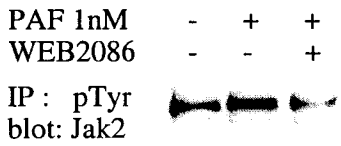
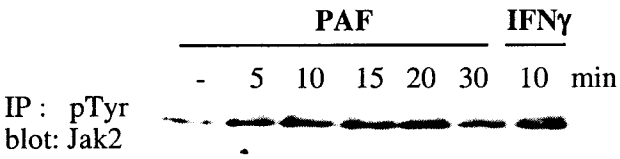

PAF 0 1 2 5 10 20 30 min
IP: Jak2
blot: pTyr
blot : Jak2

Figure 1.

A


PAF 1nM - + +
WEB2086 - - +
IP : pTyr
blot: Jak2

B


PAF
IFNγ
- 5 10 15 20 30 10 min
IP : pTyr
blot: Jak2

C

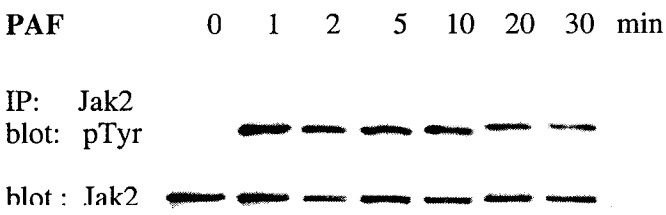

PAF 0 1 2 5 10 20 30 min
IP: Jak2
blot: pTyr
blot : Jak2

Figure 1.

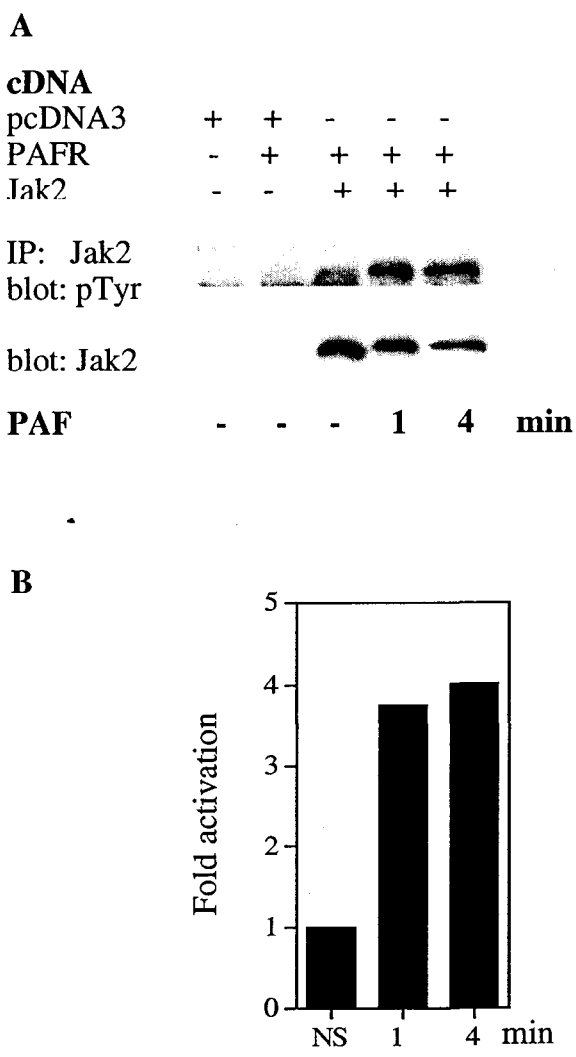
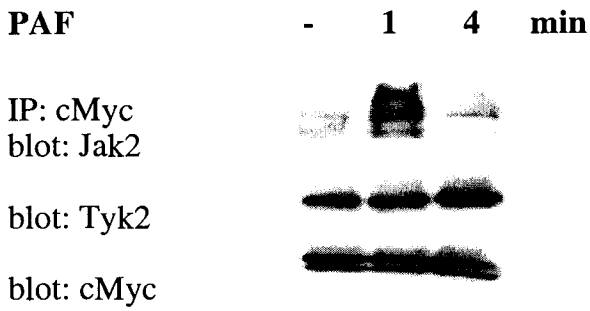

A
cDNA
pcDNA3 + + - - -
PAFR - + + + +
Jak2 - - + + +
IP: Jak2
blot: pTyr
blot: Jak2
PAF - - - 1 4 min
B
Fold activation
5
4
3
2
1
0
NS 1 4 min

Figure 2.

A


PAF
- 1 4 min
IP: cMyc
blot: Jak2
blot: Tyk2
blot: cMyc

B

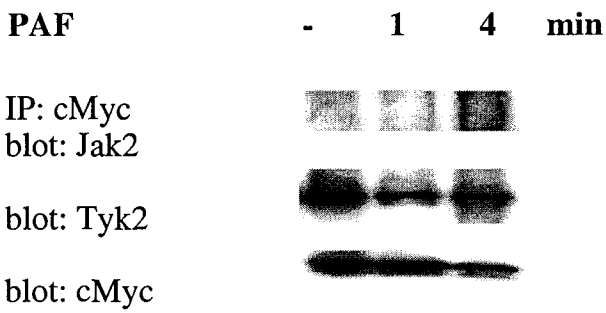

PAF
- 1 4 min
IP: cMyc
blot: Jak2
blot: Tyk2
blot: cMyc

Figure 3.

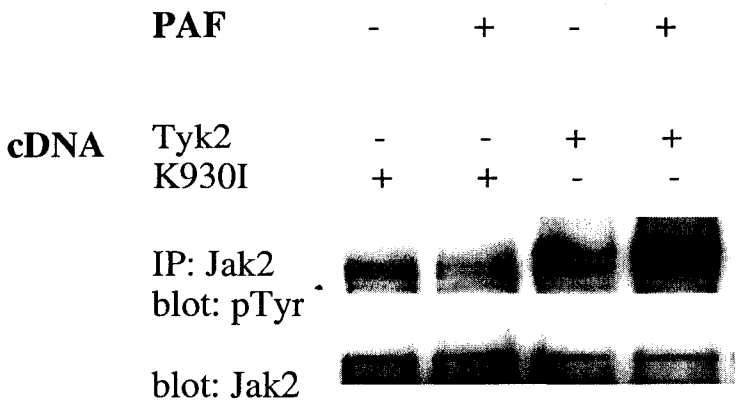
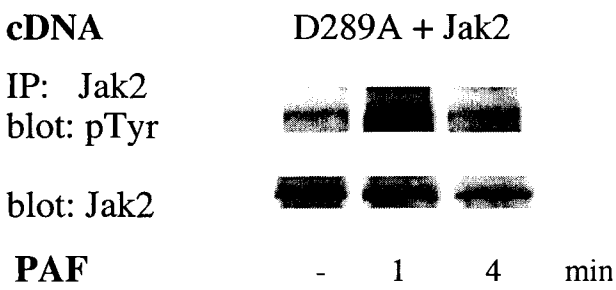


Figure 4

A


cDNA
D289A + Jak2
IP: Jak2
blot: pTyr
blot: Jak2
PAF
-
1
4
min

B

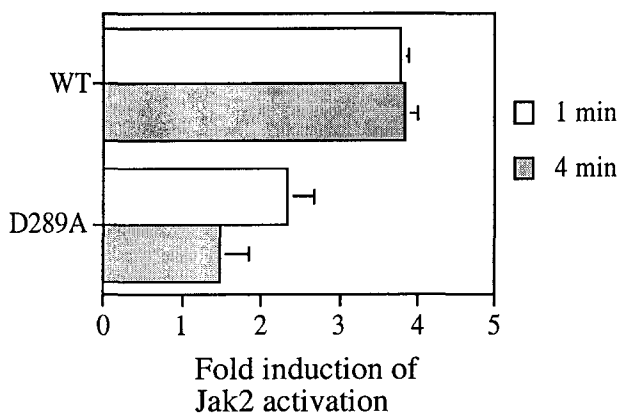
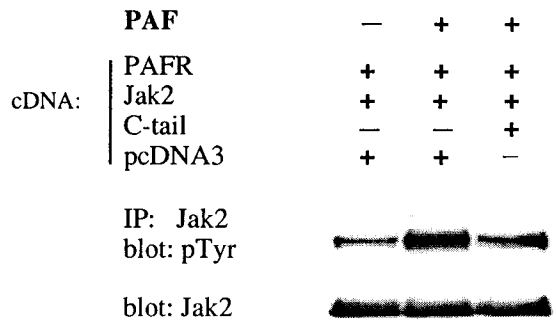

WT
D289A
1 min
4 min
0
1
2
3
4
5
Fold induction of
Jak2 activation

Figure 5

A


PAF
cDNA:
PAFR
Jak2
C-tail
pcDNA3
IP: Jak2
blot: pTyr
blot: Jak2

B

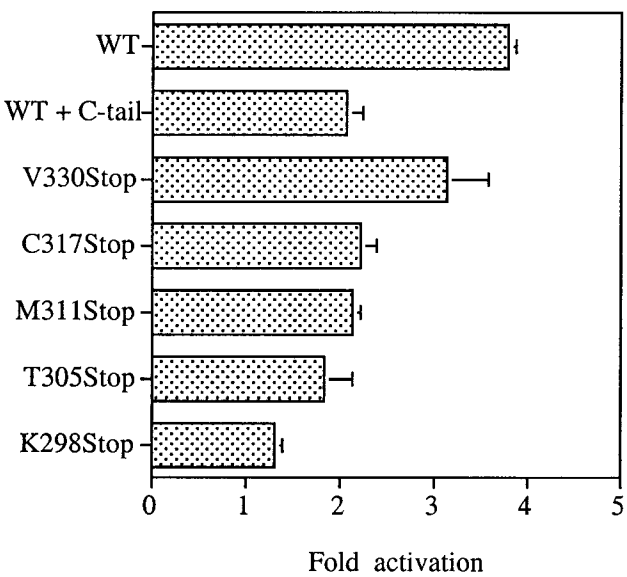

WT
WT + C-tail
V330Stop
C317Stop
M311Stop
T305Stop
K298Stop
0
1
2
3
4
5
Fold activation

Figure 6

VI. DISCUSSION

The Jak/STAT pathway was first discovered through the study of interferon signaling but is now known to participate in the signaling initiated by a wide variety of cytokines and growth factors. Whereas several GPCRs activate the Jak/STAT pathway, the mechanism(s) of this activation are still poorly defined. In the present thesis we investigated the mechanisms of PAFR-mediated activation of the Jak/STAT pathway and describe various aspects of the structure-function relationship between the receptor and components of this signaling cascade.

We showed (Chapter III and V) that PAF induces activation of tyrosine kinases Jak2 and Tyk2 in the myeloid cell line MonoMac-1 as well as in transfected COS-7 cells. This activation was receptor-mediated, as the PAF antagonist WEB2086 inhibited PAF-induced Jak2 as well as Tyk2 phosphorylation. However, the kinetics of PAF-stimulated tyrosine phosphorylation of the kinases was different. While Tyk2 activation in MonoMac-1 cells was rapid and peaked after 1-2 min of PAF treatment, Jak2 phosphorylation achieved its maximum later and was sustained for 20 minutes. In contrast to PAFR, Tyk2 activation stimulated by IFN-α in HeLa S3 cells (Colamonici et al., 1994) or by certain GPCRs, such as PAR-1 receptor for thrombin in VSMC (Madamanchi et al., 2001) or angiotensin II AT1 receptor in RASM cells (Marrero et al., 1995), achieved its maximum at 15 minutes. The kinetics of PAF-stimulated Jak2 activation was similar to that observed after angiotensin II (Marrero et al., 1995) and thrombin (Madamanchi et al., 2001) stimulation of RASM cells. Comparable activation times had also been observed for various cytokine receptors, in particular, Jak2 induction

by IL-12 in T cells (Bacon et al., 1995), and Jak1 and Jak2 phosphorylation in RASM cells upon IFN-α and IFN-γ stimulation, respectively (Marrero et al., 1995). In contrast, Jak2 activation was more transient when activated by the chemokine receptors, CCR5 and CXCR4 (Wong et al., 2001; Vila-Coro et al., 1999). Whereas, more prolonged Jak2 activation had been demonstrated in rat fetal myoblasts in response to serotonin (Guillet-Deniau et al., 1997) and in Ba/F3 pro-B-lymphocyte cells after α-MSH binding to the melanocortin MC5 receptors (Buggy, 1998). Recently, Deo and colleagues demonstrated that PAFR antagonists inhibited basic fibroblast growth factor (bFGF)-simulated Jak2 phosphorylation in HUVEC cells suggesting the involvement of the PAFR pathway in bFGF-mediated Jak2 activation (Deo et al., 2002). Under these conditions, Jak2 phosphorylation was maintained for relatively long periods of time. In COS-7 cells, transiently expressing the PAFR and Jaks, the level of PAF-induced Jak2 tyrosine phosphorylation was comparable to that in MonoMac-1 cells, with peak phosphorylation occurring at 1 min after stimulation with PAF. Jak2 activation had also been shown in COS-7 cells, transiently expressing angiotensin II AT1 receptor and the kinase (Ali et al., 2000). However, in contrast to PAFR, AT1-mediated Jak2 tyrosine phosphorylation achieved its maximum at 6 min, suggesting different mechanisms of Jak2 activation by the two GPCRs. PAF-induced increase in the level of Tyk2 tyrosine phosphorylation, in transfected COS-7 cells, was much lower than the increase seen in myeloid cells, probably due to high basal phosphorylation of Tyk2 and differences in expression levels of the kinase. Overexpression of Jaks may lead to oligomer formation and constitutive activation of the enzyme, a phenomenon often observed with overexpressed Jaks (Jiao et

al., 1996; Endo et al., 1997). This also appears to occur in both the *Drosophila hop* mutant and a subset of human acute lymphoblastic leukemias (Duhe et al., 2000). Altogether, these data may indicate cell-specific differences in GPCR-induced activation of Janus kinases or the regulation of Jak activity by different signaling pathways.

PAF-mediated activation of kinases was followed by subsequent tyrosine phosphorylation of STAT transcription factors and their translocation to the nucleus. We investigated the STAT phosphorylation status and subcellular distribution upon PAF treatment in myeloid cells as well as in a reconstituted system (Chapter III). PAF induced the tyrosine phosphorylation of STAT1, STAT2, STAT3, and STAT5. The same STATs are activated in response to SDF-1 in MOLT4 cells (Vila-Coro et al., 1999). While activation of STAT1, 2 and 3 was rapid and maximal after 15 minutes of ligand exposure, STAT5 phosphorylation was sustained for the period over 1 hour in our system. Recently, Deo and co-worker observed prolonged STAT3 phosphorylation upon PAF-stimulation in HUVEC cells with two peaks of phosphorylation (Deo et al., 2002). With a use of kinase inhibitors, the authors demonstrated that immediate STAT3 activation is dependent on Src kinase, while delayed STAT3 phosphorylation with a peak at 30 minutes requires Jak2. We did not identify kinases in myeloid cells, but in COS-7 cells, PAF-induced STAT translocation was dependent on Tyk2 or Jak2 co-transfection. In a given cell type, the identity of STATs activated could depend on time of cell exposure, concentration of the ligand and state of cell differentiation or maturation. In pre-activated peripheral blood lymphocytes, for example, IL-2 activates STAT3 and STAT5, although it only activates STAT5 in quiescent lymphocytes (Lin et al., 1995).

Next, we were interested in studying the effect of PAF on STAT localization within cells. We concentrated on STAT1 and STAT3, because PAFR promoter 1 contains putative binding sites for these transcription factors. Moreover, Ouellet and colleagues (Ouellet et al., 1994) showed that PAFR transcription is up-regulated by IFN-γ, possibly through STAT-mediated signaling. We found that STAT proteins were distributed equally in the cytosol and nucleus of transfected COS-7 cells, as has been found with transfected STATs in other systems (Herrington et al., 1999). The distribution of endogenous STATs may vary according to cell type: for example, in a T cell line, STAT3 was distributed diffusely in the cytoplasm and the nucleus of unstimulated cells, whereas STAT1 was predominantly in the cytoplasm (Zhang et al., 1995). In contrast to COS-7 cells, in MonoMac-1 cells, STAT3 was localized in the cytoplasm, and STAT1 was distributed more diffusely throughout the cell. In both COS-7 and MonoMac-1 cells, PAF stimulated translocation of the STATs into the nucleus, but in COS-7 cells, complete translocation was dependent on the co-transfection of Tyk2. It has been shown that dimerization of STAT proteins is prerequisite of their interaction with importin-α5 and the small GTP-binding protein Ran, and subsequent nuclear translocation. However, tyrosine phosphorylation alone is not sufficient for STAT translocation (McBride et al., 2002). Moreover, the kinetics of STAT nuclear import are very variable, depending on the STAT and its serine-phosphorylation state (Zhang et al., 1995; Beadling et al., 1996). It is not unreasonable to suggest that Tyk2 induces not only STAT tyrosine phosphorylation, but also activates directly or indirectly serine/threonine kinases thus promoting STAT nuclear import and increasing transcriptional activity.

The paradigm of STAT activation by cytokine receptors is that STATs are transiently recruited to the receptor complex via binding to specific phosphotyrosine residues through their SH2 domain (Darnell, 1997). However, certain receptors, such as receptors for GM-CSF, EPO and prolactin can interact with STATs independently of receptor phosphotyrosine residues (rev. Pellegrini and Dusanter-Fourt, 1997). STAT5 activation by gp130 required direct interaction with Jak2 (Fujitani et al., 1997). Phosphotyrosine residues of Jak2, but not those of the receptor, were found to be critical for recruiting STAT1 to the AT_1 receptor complex (Ali et al., 2000). In skeletal muscle myoblasts, the 5-HT_{2A} receptor co-precipitates with Jak2 and STAT3 (Guillet-Deniau et al., 1997), but the relevant tyrosine residues have not been determined. Tyk2 and STAT3 form a complex with the B2 receptor in response to bradykinin stimulation (Hong et al., 2000). Recently, Sayeski and colleagues discovered a Jak2 motif (^{231}YRFRR) which is required for the angiotensin II-dependent nuclear translocation of STAT1 in COS-7 cells (Sayeski et al., 2001). Mutation of this motif to ^{231}FAAAA results in an enzyme that autophosphorylates but fails to associate with the AT1 receptor. Interestingly, STAT1 was nevertheless phosphorylated in cells expressing Jak2 ^{231}FAAAA mutant. It appears that the AT1 receptor-Jak2 co-association is obligatory for STAT1 translocation and subsequent gene transcription (Sayeski et al., 2001). Similarly, to the AT1 receptor (Marrero et al., 1995), PAFR activates Jak2 and Tyk2. However, in the present thesis we did not investigate the PAFR-STAT interaction. The PAF receptor contains a tyrosine residue in the first intracellular loop as well as in its C-terminal tail. It will be interesting therefore to explore whether STAT activation by PAFR involves a direct interaction with the PAFR or through Tyk2 or Jak2.

To investigate the role of the Jak/STAT pathway in regulation of gene expression, we analyzed PAFR promoter activation using the luciferase reporter gene assay. Two distinct promoters (PAFR promoter 1 and 2) are involved in regulating the expression of human PAFR gene. The two PAFR transcripts differ only in the untranslated 5' region (Mutoh et al., 1993). In our assay we used the PAFR promoter construct that contained two putative sites for STAT binding. Our results indicate that the sequence from -157 to +37 (p0.16Luc) can functionally direct the expression of the luciferase gene. In cells transfected with PAFR and p0.16Luc, there was significant increase of luciferase activity in response to PAF in the presence of Tyk2. Interestingly, Jak2, when co-transfected, could not induce PAFR transcription. On the other hand, we observed STAT3, but not STAT1, translocation in cells overexpressing Jak2 (Lukashova and Stankova, unpublished results). One could assume that STAT3 binding site located at position –49 to –27 is not functional or, alternatively, the other STAT-binding site (at position –86 to –72) should also be engaged to induce promoter activation. Indeed, the co-transfection of STAT1 or STAT3 with Tyk2 and PAFR potentiated luciferase activity. Further mutagenesis will be necessary to elucidate the relevant importance of each STAT-binding site. It is well known that STAT proteins may form complexes with other transcription factors to induce gene expression. For, example, activation of the MHC class II transactivator CIITA by IFNγ required cooperative interaction between USF-1 and STAT1 (Muhlethaler et al., 1998). IFNα induces the formation of a complex comprising STAT2, STAT1 and IRF9 that binds IFNα-stimulated response element (ISRE) (Darnell, 1997). We could not exclude the possibility that other transcription factors, such as STAT2, activated by Tyk2, are not activated by Jak2.

The consensus binding sites for NF-κB were identified in the promoter region for PAFR transcript 1 (Mutoh et al., 1994). These sites were necessary for PAF-mediated up-regulation of its receptor in a human stomach cancer cell line. NF-κB was also involved in the up-regulation of PAFR transcription in response to TNF-α in MonoMac-1 cells (Dagenais et al., 1997) and to mechanical stretch in pulmonary artery smooth muscle cells (Chaquour et al., 1999). We show that PAF can stimulate PAFR expression in the absence of these NF-κB consensus sites. One could expect that in a given cell type, different signaling pathways may participate in the regulation of a particular gene. It has been shown that PAF-stimulated NF-κB activity in human monocytes was sensitive to PTX treatment and inhibitors of PI3 kinase, whereas in CHO cells, expressing the cloned PAF receptor, it was Cholera toxin that inhibited NF-κB activation (Ye et al., 1996). Transforming growth factor-β up-regulates PAFR transcript 1 in leukocytes (Parent and Stankova, 1993; Yang et al., 1997), but not in a human stomach cancer cell line (Mutoh et al., 1994a). In addition, transcriptional factors might modulate the transcriptional activity of each other (Greenlund et al., 1995; John et al., 1996). Depending on the composition of the STAT-containing complex bound to a given DNA, activation or inhibition of transcription might occur. Since both transcripts of PAFR are present in several cell types, careful analysis of each promoter will be needed to shed light on the relative importance of different signaling pathways and transcription factors in the regulation of the PAFR gene.

We next sought to determine the mechanisms of Jak/STAT activation stimulated by PAF. We analyzed Tyk2-dependent PAFR transcription and Jak2 tyrosine phosphorylation induced by PAF in COS-7 cells (Chapter III and V). Previous reports

have demonstrated that the PAFR receptor can interact with multiple G-proteins, leading to simultaneous stimulation of distinct signaling pathways. PAF-induced cellular responses, such as calcium mobilization and IP production, are mediated by both PTX-sensitive G-proteins, G_i and G_o, in addition to other PTX-insensitive G-proteins, such as G_q (Honda et al., 1994; Ali et al., 1994). Although involvement of some G-proteins can be monitored with the help of inhibitors, G-protein uncoupled mutants represent an excellent tool to investigate G-protein-independent signaling. We found that PAF mutants with impaired capacity to signal via G-proteins, D289A, D63N, and Y293A, were able to induce Tyk2-dependent PAFR promoter 1 activation. Similarly to the WT receptor, the G-protein uncoupled PAFR mutant D289A induced Jak2 phosphorylation. These results provide direct evidence of G-protein independent Jak/STAT signaling by PAFR. Similarly to PAFR, other GPCRs may also transduce signals independently of G-protein activation. Stimulation of the Jak2/STAT3 pathway by CCR2B and CXCR4 was not inhibited by pertussis toxin, suggesting a Gαi-independent action. (Mellado et al., 1998; Vila-Coro et al., 1999). G-protein-independent STAT activation has been also proposed for serotonin 5-HT_{2A} receptor (Guillet-Deniau et al., 1997) and angiotensin II AT1 receptor (Ali et al., 2000). In contrast, Hunt and co-workers demonstrated that angiotensin II–stimulated STAT3 tyrosine phosphorylation in neonatal rat ventricular myocytes occurs through a $G\alpha_q$-PLC-PKC–mediated pathway (Hunt et al., 1999). Altogether, these data suggest that different G-proteins might be upstream of Jak/STAT signaling cascade induced by certain GPCRs, and/or alternatively, the Jak/STAT pathway might be direct and G-protein-independent in case of other GPCRs. One could not exclude the role of G-proteins in regulating Jak/STAT activation in a yet unknown way.

Very little is known about motifs within GPCRs important for Jak activation. CCR2B receptor with mutation of conserved tyrosine residue at position 139 in the second intracellular loop failed to induce Jak2 activation (Mellado et al., 1998). In the corresponding DRY motif, the PAFR has phenylalanine instead of tyrosine residue. This position would therefore be not important for PAFR-induced Jak2 activation. The AT1 receptor lacking the YIPP motif in the C-terminus is unable to stimulate ligand-dependent phosphorylation of Jak2 (Ali et al., 1997). However, PAFR does contain a tyrosine residue at the position 309 in its C-tail, and we therefore investigated the role of this region for the activation of Jak2 and PAF-induced Tyk2-dependent PAFR promoter activation. We found that the receptor C-terminal deletion mutants T305Stop, M311Stop and C317Stop were still able, although less effectively than WT, to activate Jak2. However, partial inhibition of Jak2 phosphorylation was observed when the minigene encoding the 46 C-terminal residues was co-transfected, indicating that the C-tail of the receptor does represent an important domain in PAFR-mediated Jak2 activation. Interestingly, M311Stop as well as the co-transfection of the minigene encoding the C-terminus of the receptor, showed reduced Tyk2-dependent PAFR promoter activation. On the other hand, inositol phosphate production induced by PAF was not changed under these circumstances (Chen et al., 2002; Chapter IV), indicating that other receptor functions were not affected. One could propose that the C-terminus of the receptor is critical not only for Jak2, but also for Tyk2 activation, or alternatively, it serves as a docking site for other proteins that might modulate kinase activity. While, originally, the Jak2 interaction with the AT1 receptor was thought to be direct, subsequent studies

revealed that antibodies against SHP-2 phosphatase could block Jak2/AT1 receptor interaction. SHP-2 binds to the same YIPP motif in the C-terminus of the AT1 receptor that was required for Jak2 association and appears to regulate Jak2 activation and association with the receptor (Marrero et al., 1998). The ITIM motif in the C-terminus of the bradykinin B2 receptor is required for its interaction with SHP-2 phosphatase in primary culture renal mesangial cells. Association between this receptor and phosphatase appears to be necessary for the anti-mitogenic effect of bradykinin (Duchene et al., 2002). Despite the absence of YIPP or ITIM consensus motifs in the C-terminus of the PAFR, we could not exclude the possibility that phosphatases are also involved in regulation of PAF-stimulated Jak kinase activity.

Our results showed a significant inhibitory effect ($p<0.05$) of minigenes encoding the second intracellular loop or C-terminus of the receptor on PAF-stimulated Tyk2-dependent PAFR transcription. Co-transfection of two minigenes (1-in + 3-in) or (2-in + 3-in) also resulted in a significant inhibition of reporter gene activation ($p<0.01$). Comparative statistical analysis showed no significant differences between the cells transfected with a minigene encoding the first intracellular loop (1-in) and mixture of minigenes (1-in and 3-in). However, significant difference ($p<0.05$) was observed between cells transfected with the minigene encoding the third intracellular loop (3-in) and mixture of two minigenes (1-in + 3-in). In addition to the C-terminus of GPCRs, the intracellular second and third loops have been shown to interact with arrestins (Bennett et al., 2000; Van Koppen et al., 1995). Recent reports have suggested a novel role for the β-arrestin in recruiting and activating c-Src (Luttrell et al., 1999). This recruitment of β-arrestin and c-Src resulted in GPCR-mediated ERK activation, as demonstrated for the

neurokinin-1 (Nk-1) receptor (DeFea et al., 2000) and β_2 AR receptor (Luttrell et al., 1999; Miller et al., 2000). Recently, our group found that PAFR could associate with arrestin-2 and –3 (Chen et al., 2002). PAF-induced arrestin translocation was dependent on the motif DPXXYP in the seventh transmembrane domain of PAFR and C-terminal region spanning residues from 318 to 330. The fact that the PAFR C-terminal deletion mutants and D289A, which do not induce arrestin translocation, nevertheless induced Jak2 phosphorylation suggests that PAF-mediated Jak2 activation is independent of arrestins. The PAFR mutant D289A also has an impaired capacity to internalize (Le Gouill et al., 1997). However, it induced PAFR transcriptional activation and Jak2 tyrosine phosphorylation, indicating that both Jak2 activation and Tyk2-dependent signaling are internalization-independent. The inhibitory effect of minigenes encoding intracellular loops on PAFR transcriptional activation could be explained by a possible interference with receptor conformation responsible for proper Tyk2 activation. We could not exclude the possibility that co-transfection of two minigenes may also impair STAT interaction with the receptor resulting in a more profound inhibition.

The most intriguing finding was the complete failure of mutant E51A of PAFR to induce PAF-stimulated PAFR transcription in the presence of Tyk2 ($p<0.001$). Reduced PAFR promoter activation was also observed in case of G134D and L43F mutants ($p<0.05$) whereas other receptor functions remained unaffected. Janus kinase binding and activation induced by cytokine receptors appear to co-localize. Mutation of leucine or isoleucine as well as acidic cluster to alanine residues in IFNaR1 disrupts Tyk2 binding and subsequent STAT2 phosphorylation (Yan et al., 1996). This is also the case of

chemokine CCR2B receptor where substitution of critical tyrosine residue at position 139 resulted in loss of Jak2 association and activation (Mellado et al., 1998). Similarly to the IFNaR1 receptor, mutation of glutamic acid E51 to alanine in the PAF receptor results in the complete loss of PAF-stimulated Tyk2-dependent function. In contrast to the IFNaR1 receptor, changes in basic environment (KK4748AA or G134D) in the PAFR only partially disrupts Tyk2-dependent PAFR transcription. We could hypothesize that despite unaltered Tyk2 binding, mutant receptors may have changed their conformation and thus fail to activate the kinase or, alternatively, mutations mask an important site, for example, for STAT binding. PAFR contains a tyrosine residue (Y44) in its first intracellular loop. One could assume that this residue serves as a docking site for STAT proteins. Mutations of neighboring amino acids, for example L43F or K4748AA, could results in a receptor incapable of binding these transcription factors and as a consequence be unable to induce reporter gene activation. In this way, the mutation of a valine residue at position 467 in the IFNaR1 receptor, in close proximity to the STAT2 binding site, inhibited STAT2 co-immunoprecipitation and IFN-α signaling (Krishnan et al., 1998).

In addition to PAFR, two other GPCRs, angiotensin II AT1 and bradykinin B2 (B2R) receptors are known to induce Tyk2 activation (Marrero et al., 1995; Ju et al., 2000). However, AT1 receptor bound Jak2, but not Tyk2 (Marrero et al., 1995). Tyk2 could form a complex with B2R receptor in a ligand-dependent manner (Ju et al., 2000), but this association has not been studied in detail. Association of chemokine receptors with Jak family members has also been reported (Mellado et al., 1998; Vila-Coro et al., 1999). In all cases, association of Jaks with GPCRs was ligand- and time-dependent, although some basal interaction was observed, in some cases, in quiescent cells (Vila-

Coro et al., 1999). Similarly to other GPCR, Jak2 association with the PAF receptor was transient and ligand-dependent (Chapter V). However, it was observed only in the presence of active Tyk2. In contrast, PAFR constitutively associates with Tyk2, independently of agonist binding. Tyk2 is also co-precipitated with G-protein uncoupled mutants and co-localized with PAFR in myeloid and transfected cells independently of ligand exposure, again, indicating constitutive association of the kinase with the receptor. Thus, PAFR-Tyk2 interaction resembles the situation with cytokine receptors, for example, the interaction of Tyk2 with IFNaR1 (Colamonici et al., 1994; Colamonici et al., 1994a).

Jaks can function as scaffolding proteins, recruiting STATs and other proteins to the receptor complex (Fujitani et al., 1997; Ali et al., 2000; Duhe et al., 2001). Since Jak2 association was not observed in the presence of the dominant negative mutant of Tyk2, K930I (Chapter V), we could propose that phosphorylated Tyk2 would serve as a docking site for Jak2. It is unlikely that Jak2 is recruited through tyrosine residues in the C-terminus of PAFR, since its deletion still permits Jak2 activation. It would therefore be interesting to investigate whether PAF stimulation induces a direct association between the two kinases. Alternatively, other proteins could serve this function. PAF stimulates c-Src phosphorylation (Dhar and Shukla, 1994) and it had been shown that angiotensin II-induced association of Jak2 and c-Src requires the N-terminus of Jak2 and the SH2 domain of c-Src kinase (Sayeski et al., 1999). Recently, Deo and co-workers reported that both kinases are involved in PAF-induced STAT3 phosphorylation (Deo et al., 2002). Adapter proteins APS and SH2-B have been shown to be activated in response to GH,

IFN-γ, LIF, EPO, IL-3 and IL-5 (O'Brien et al., 2002; Wakioka et al., 1999; Iseki et al., 2000). Sh2-B directly activates Jak2 thus providing a powerful positive feedback mechanism for ligands that stimulate Jak2. Moreover, this protein binds phosphorylated Jak1 and Jak2 and may serve as an adapter for these kinases, recruiting other proteins, through kinases phosphorylated on tyrosine residues to receptor-Jak-SH2-B complexes (O'Brien et al., 2002). In contrast, APS interacts with three Janus family members: Jak1, Jak2 and Jak3, and negatively regulates Jak1 and Jak2. Phosphotyrosine residues on APS may provide docking sites for downstream molecules that decrease Jak kinase activity (Iseki et al., 2000; O'Brien et al., 2002). Regulatory function of such scaffolds could be proposed in modulation of Janus kinase activity induced by GPCRs.

Tyk2 kinase activity has also been shown to be required for Jak2 trans-phosphorylation. PAF-induced Jak2 phosphorylation was inhibited by co-transfection of dominant-negative mutant Tyk2, K930I. Similarly to cytokine receptors, angiotensin II stimulates Jak2 phosphorylation at tyrosine residues Y1007 and 1007 in vascular smooth muscle cells, and Jak2 activation was inhibited by overexpression of dominant-negative PKCδ and Pyk2 (Frank et al., 2002). Phosphorylation of regulatory tyrosine residues within enzyme activation loops is a well-known mechanism of regulation of Janus kinase activity in response to cytokine stimulation. Jak1 was shown to be obligatory in initiation of phosphorylation and activation of Tyk2 in the IFN-α/β receptor complex (Gauzzi et al., 1996). In the case of the IFN-γ receptor complex, Jak2 serves as the initiator kinase that phosphorylates Jak1 as part of the signaling cascade (Briscoe et al., 1996). A dominant-negative Jak1 prevents the activation of Tyk2 after IFN-β stimulation in 293T cells (Briscoe et al., 1996), and an inactive Jak2 blocks the activation of Jak1 in response

to IFN-β receptor stimulation (Krishnan et al., 1997). Similarly to cytokine receptors, certain GPCRs, for example, the chemokine receptors, form dimers upon ligand binding (Vila-Coro et al., 1999). Thus, Jak activation could resemble the mechanism proposed for cytokine receptors, which is kinase trans-phosphorylation upon receptor dimerization. All the GPCRs, which have been shown to activate the Jak/STAT pathway, except CCR2B, are known to induce activation of several Jaks. However, kinase interdependence has not been investigated. In contrast, PAFR potentially form oligomers independently of ligand binding (Le Gouill et. al., 1999). Thus, Tyk2 activation is very likely the result of conformational changes upon ligand stimulation, given that Tyk2 is constitutively associated with the receptor, whereas Jak2 recruitment and activation to the receptor complex required the presence of another family member, Tyk2.

A number of cytokine receptor-binding domains within Jak family kinases have now been characterized. In the case of Jak2, which associates with a wide variety of receptors, binding to the growth hormone receptor appears to require the entire amino-terminal half of the protein (JH3-7) (Tanner et al., 1995). In contrast, binding to the GM-CSF receptor requires only the JH6 and JH7 domains (Zhao et al., 1995). Tyk2 binding domain on IFNaR1 receptor spans the amino-terminal half of the protein, although the individual JH3 and JH6 domains show independent binding (Yan et al., 1998). We used constructs which contained only the JH6 and JH7 regions (amino acids 1-262) or only JH3-JH5 regions (262-601) or both (1-601) of Tyk2 to investigate the regions of Tyk2, which could associate with the PAFR. Our results show that each of the truncated proteins containing only one binding domain can associate with PAFR. These results

suggest a binding pattern similar to that of the interferon-α receptor (Yan et al., 1998; Colamonici et al., 1994).

To further characterize the receptor-kinase association, we performed in vitro binding experiments of Tyk2 with GST-fusion proteins of PAFR and co-immunoprecipitation of the kinase using the receptor deletion mutants (Chapter IV). Our results show that PAFR contains multiple binding sites for the kinase. We observed binding of WT Tyk2 to a GST-fusion protein bearing the second and third intracellular loops and the C-terminus of the receptor. Interestingly, the PAFR second and third intracellular loops do not contain tyrosine residues, suggesting that tyrosine phosphorylation is not a requirement for PAFR-Tyk2 binding. However, we could not exclude the possibility that phosphorylation of tyrosine residues, Y44 or Y309, is still necessary for STAT binding to the PAFR. In the case of cytokine receptors, a given STAT may bind tyrosine-phosphorylated motifs (Pellegrini and Dusanter-Fourt, 1997), however, some receptors, such as those for growth hormone, IL-3, IL-5, G-CSF can activate STATs even in the absence of tyrosine residues in the receptor intracellular domain (Caldenhoven et al., 1995; Wang et al., 1995). Similarly, in the angiotensin II AT1 receptor, substitution of all tyrosine residues to phenylalanine did not affect receptor-Jak2-STAT1 complex formation (Showkat et al., 2000). In our co-immunoprecipitation studies, the PAFR mutant, T305Stop, lacking the 37 C-terminal residues, still precipitated Tyk2. While the C-terminus of the receptor is not essential for kinase binding, it is evident that that its presence is necessary for proper Tyk2 activation, since M311Stop showed a significant decrease ($p<0.05$) in PAF-stimulated Tyk2-dependent PAFR transcription. In contrast to the AT1 receptor (Marrero et al., 1997), the

tyrosine residue in the PAFR C-tail is not required for the receptor-kinase interaction, since the mutant T305Stop, lacking this residue, binds Tyk2 similarly to the WT receptor. It is very likely that the second intracellular loop represents the structural requirement for both binding and activation of the kinase. Removal of either three or six residues in this loop significantly diminished the co-immunoprecipitation of the kinase and disrupted PAFR promoter activation. In contrast to IFNaR1-Tyk2 interaction, that required hydrophobic and acidic amino acids (Yan et al., 1996), a basic environment (amino acids 131-133) in the PAFR seems to be critical for its association with the kinase. These finding are in agreement with our theoretical model of the PAFR-Tyk2 interaction (Lukashova et al., 2000). We predicted that Tyk2 binding sites would localize in the receptor first and second intracellular loops. However, deletion of residues within the first intracellular loop of the receptor resulted only in a partial decrease of Tyk2 binding, indicating that other receptor motifs might be involved. Therefore, we could not exclude the possibility that PAFR intracellular loops might form a conformational binding pocket for the kinase. Mutation or deletion of residues critical for the binding, or in close proximity, to the binding site may result in a receptor conformation incapable of binding and/or activating the kinase.

VII. CONCLUSION

The present thesis shows the activation of the Jak/STAT signaling cascade by the platelet-activating factor receptor and its role in regulation of PAFR transcription. We found that in COS-7 cells, co-transfection of Tyk2 with PAFR significantly augmented PAFR promoter 1 activation. In addition, co-transfection of either STAT1 or STAT3 potentiated PAF-induced PAFR transcription. To confirm the involvement of STATs in PAFR transcription, it would be interesting to mutate putative STAT-binding sites within the PAFR promoter. Luciferase assays as well as EMSAs could be performed in COS-7 and MonoMac cells employing the mutant promoter. We demonstrated that PAF induced tyrosine phosphorylation of STAT2 and STAT5 in MonoMac cells, however, their role in PAFR signaling requires further investigation. In addition, the present thesis we did not investigate PAFR-STAT interaction. Co-immunoprecipitation studies followed by Western blot analysis could be performed to explore whether STATs directly interact with the PAFR or through Tyk2 or Jak2.

Our work provides novel insight into the mechanism of Jak2 and Tyk2 activation and receptor co-association. Specifically, it defines the mechanism of kinase activation as G-protein and arrestin/internalization independent. It determines that the Tyk2-PAFR interaction is constitutive and independent of ligand. On the other hand, the Jak2-PAFR association depends on agonist exposure and occurs only in the presence of active Tyk2. A direct association between the two kinases in response to PAF could be investigated by co-immunoprecipitation and Western blot analysis using differentially tagged kinases. In addition, Tyk2 kinase activity was found to be required for Jak2 phosphorylation. It would be interesting to further study kinase hierarchy in PAFR signaling employing

kinase deficient cell lines or kinases with mutated tyrosine residues within their activation loop.

PAF-induced Jak/STAT pathway might be negatively regulated by phosphatases or SOCS proteins. Our preliminary data show that PAF modulates SOCS3 mRNA and protein expression in human monocytes. In addition, Tyk2-dependent PAFR transcription was inhibited by co-transfection of SOCS2 or SOCS3, but not SOCS1 cDNA. Further investigation of possible negative regulators of PAFR signaling is required.

Importantly, this work establishes the role of the C-terminus of the receptor both, in Jak2 activation and Tyk2-dependent PAFR promoter 1 activation and the region of residues 127-133 within the second intracellular loop of PAFR as critical for Tyk2-binding and signaling. Introduction of alanine residues into the basic environment (KK4748A) or change of a neutral glycine to an aspartate residue near the basic cluster (G134D) of the receptor diminished Tyk2-dependent PAFR transcription. Most significant, is the observation that substitution of glutamic acid at position 51 to alanine results in the complete loss of Tyk2-dependent, PAF-mediated PAFR transcription. However, we did not investigate whether these changes in the PAFR result in diminished Tyk2/Jak2 or STAT activation. To further characterize the mutant PAF receptors, it would be interesting to study whether changes in the receptor affect Tyk2 or Jak2 phosphorylation itself or other proteins of the signaling cascade (STATs, SOCSs, phosphatases).

In summary, our results extend the model of cytokine receptor signaling to the lipid mediator, PAF, and highlight several aspects of structure-function relationships

between a member of the GPCR family, PAFR, and components of the Jak/STAT signal transduction pathway.

ACKNOWLEDGMENTS

I'd like to thank Dr. Jana Stankova and Dr. Marek Rola-Pleszczynski for the great opportunity to do research in their laboratories and present results in conferences, for the patience on the way of new results, for the emotional support during all these years...

I'd like also to thank all the professors of the Immunology Program: Dr. Dupuis, Dr. Dubois, Dr. Fullop, Dr. Fournier, for their interesting courses and discussions.

I'd like to thank Dr. Natalie Rivard, Dr. Gilles Dupuis and Dr. Paul Naccache for the evaluation of my thesis.

Un GRANDE MERCI Denis Gingras, Sivie Turcotte, Leonid Volkov, pour vos conseils and votre contribution á nos experiences, votre support et l'attention d'un jour á l'autre.

Un GROS MERCI Melanie, Genevieve, Stephan, Karina, Aline, Maryse, Marie-Hélène, Charles, Rémi, Denis, Christian, Amélie pour votre gentilesse, sourire, discussions....

REFERENCES:

Adams, T.E., Hansen, J.A., Starr, R., Nicola, N.A., Hilton, D.J., Billestrup, N. Growth hormone preferentially induces the rapid, transient expression of SOCS-3, a novel inhibitor of cytokine receptor signaling. J. Biol. Chem. 273: 1285-1287, 1998.

Ali, H., Richardson, R.M., Tomhave, E.D., DuBose, R.A., Haribabu, B., Snyderman, R. Regulation of stably transfected Platelet-activating factor receptor in RBL-2H3 cells. J. Biol. Chem. 269: 24557-24563, 1994.

Ali, M.S., Sayeski, P.P., Dirksen, L.B., Hayzer, D.J., Marrero, M.B., Bernstein K.E. Dependence on the motif YIPP for the physical association of Jak2 kinase with the intracellular carboxyl tail of the angiotensin II AT1 receptor. J. Biol. Chem. 272: 23382-23388, 1997.

Ali, M.S., Sayeski, P.P., Bernstein, K.E. Jak2 acts as both a STAT kinase and as a molecular bridge linking STAT1 to the angiotensin II AT1 receptor. J. Biol. Chem. 275: 15586-15593, 2000.

Bacon, C. M., McVicar, D.W., Ortaldo, J. R., Rees, R.C., O'Shea, J. J., Johnston, J.A.. Interleukin 12 (IL-12) induces tyrosine phosphorylation of JAK2 and TYK2: differential use of Janus family tyrosine kinases by IL-2 and IL- 12. J Exp Med 181:399-404, 1995.

Barzaghi, G., Sarau, H.M., Mong, S. Platelet-activating factor-induced phosphoinositide metabolism in differentiated U-937 cells in culture. J. Pharmacol. Exp. Ther. 148: 559-566, 1989.

Bazan, N.G., Squinto, S.P., Braquet, P., Panetta, T., Marcheselli, V.L. Platelet-activating factor and polyunsaturated fatty acids in cerebral ischemia or convulsions: intracellular PAF-binding sites and activation of a fos/jun/AP-1 transcriptional signaling system. Lipids 26: 1236-1242, 1991.

Beadling, C., Ng, J., Babbage, J.W., Cantrell, D.A. Interleukin-2 activation of STAT5 requires the convergent action of tyrosine kinases and a serine/threonine kinase pathway distinct from the Raf1/ERK2 MAP kinase pathway. EMBO J. 15: 1902-1913, 1996.

Bennett, T.A., Maestas, D.C., Prossnits, E.R. Arrestin binding to the G protein-coupled N-formyl peptide receptor is regulated by the conserved "DRY" sequence J. Biol. Chem. 275, 24590-24594, 2000.

Benveniste, J., Henson, P.M., Cochrane, C.G. Leukocyte-dependent histamine release from rabbit platelets. The role of IgE, basophils, and a platelet-activating factor. J. Exp. Med.136: 1356-1377, 1972.

Benveniste, J., Tence, M., Varenne, P., Bidault, J., Boullet, C., Polonsky, J. Semi-synthesis and proposed structure of platelet-activating factor (P.A.F.): PAF-acether an

alkyl ether analog of lysophosphatidylcholine. J.C.R.Acad. Sci.paris Ser. D 289: 1037-1040, 1979.

Binari, R. and Perrimon, N. Stripe-specific regulation of pair-rule genes by hopscotch, a putative Jak family tyrosine kinase in Drosophila. Genes Dev. 8: 300-312, 1994.

Bhattacharya, S., Eckner, R., Grossman, S., Oldread, E., Arany, Z., D'Andrea, A., Livingston, D.M. Cooperation of STAT2 and p300/CBP in signaling induced by interferon-alpha. Nature 383: 344-347, 1996.

Bito, H. Platelet-activating factor (PAF) receptor in rat brain: PAF mobilizes intracellular Ca^{2+} in hyppocampcal neurons. Neuron 9: 285-294, 1992.

Bito, H., Honda, Z., Nakamura, M., Shimizu, T. Cloning, expression and tissue distribution of rat platelet-activating-factor-receptor. Eur. J. Biochem. 221: 211-218, 1994.

Blank, M.L., Snyder, F., Byers, L.W., Brooks, B., Muirhead, E.E. Antihypertensive activity of an alkyl ether analog of phosphatidylcholine. Biochem. Biophys. Res. Commun. 90: 1194-1200, 1979.

Braquet, P., Esanu, A., Buisine, E., Hosford, D., Broquet, C., Kotlai, M. Recent progress in ginkgolide research. Med. Res. Rev. 11: 295-355, 1991.

Brass, L.F., Wooikalis, M.J., Manning, D.R. Interactions in platelets between G proteins and the agonists that stimulate phospholipase C and inhibit adenylyl cyclase. J. Biol. Chem. 263: 5348-5355, 1988.

Briscoe, J., Rogers, N.C., Witthuhn, B.A., Watling, D., Harpur, A.G., Wilks, A.F., Stark, G.R., Ihle, J.N., Kerr, I.M. Kinase-negative mutants of Jak1 can sustain interferon-_-inducible gene expression but not an antiviral state. EMBO J. 15: 799-809, 1996.

Buggy, J.J. Binding of α-melanocyte-stimulating hormone to its G-protein-coupled receptor on B-lymphocytes activates the Jak/STAT pathway. Biochem. J. 331: 211-216, 1998.

Bussolino, F. and Camussi, G. Platelet-activating factor produced by endothelial cells. A molecule with autocrine and paracrine properties. Eur. J. Biochem. 229: 327-337, 1995

Cacalano, N.A., Migone, T.S., Bazan, F., Hanson, E.P., Chen, M., Candotti, F., O'Shea, J.J., Johnston, J.A. Autosomal SCID caused by a point mutation in the N-terminus of Jak3: mapping of the Jak3-receptor interaction domain. EMBO J.18: 1549-1558, 1999.

Calcerrada, M.C., Calatan, R.E., Pérez-Alvarez, M., Miguel, B.G., Martinez, A.M. Platelet-activating factor stimulation of p125(FAK) and p130(Cas) tyrosine phosphorylation in brain. Brain Res. 835: 275-281, 1999.

Caldenhoven, E., van Dijk, T., Raaijmakers, J.A., Lammers, J.W., Koenderman,L., Groot, R.P. Activation of the STAT3/acute phase response factor transcription factor by interleukin-5. J. Biol. Chem. 270: 25778-25784, 1995.

Cao, X., Shores, E.W., Hu-Li, J., Anver, M.R., Ketsall, B.L., Russell, S.M., Drago, J., Noguchi, M., Grinber, A., Bloom, E.T. et al. Defective lymphoid development in mice lacking expression of the common cytokine receptor γ chain. Immunity 2: 223-238, 1995.

Carlson, S., Chatterjee, T.K., Fisher, R.A. The third intracellular domain of the Platelet-activating factor receptor is critical determinant in receptor coupling to phosphoinisitide phospholipase C-activating G proteins. J. Biol. Chem. 271: 23146-23153, 1996.

Carlson, S., Chatterjee, T.K., Murphy, K.P., Fisher, R.A. Mutation of a putative amphipathic α-helix in the third intracellular domain of the Platelet-activating factor receptor disrupts receptor/G protein coupling and signaling. Mol. Pharm. 53: 451-458, 1998.

Cassals-Stenzel, J., Muacevic, G., Weber, K.H. Pharmacological actions of WEB 2086, a new specific antagonist of platelet activating factor. J. Pharmacol. Exp. Ther. 241: 974-981, 1987.

Chao, W. and Olson, M.S. Platelet-activating factor: receptors and signal transduction. Biochem. J. 292: 617-629, 1993.

Chaqour, B., Howard, P.S., Richards, C.F., Macarak, E.J. Mechanical stretch induces platelet-activating factor receptor gene expression through the NF-kappaB transcription factor. J. Mol. Cell. Cardiol. 31: 1345-1355, 1999.

Chen, Z., Le Gouill, C., Dupre, D., Rola-Pleszczynski, M., Stankova J. Agonist-induced internalization of the platelet-activating factor receptor is dependent on arrestins but independent of G-protein activation. Role of the C-terminus and the D/NPXXY motif. J. Biol. Chem. 277: 7356-7362, 2002.

Chung, C.D., Liao, J., Lui, B., Rao, X., Jay, P., Berta, P., Shuai, K. Specific inhibition of STAT3 signal transduction by PIAS3. Science 278:1803-1805, 1997.

Clapham, D.E. G protein βγ subunits. Annu. Rev. Pharmacol. Toxicol. 37:167-203, 1997.

Coffer, P.J., Geijen, N., M'Rabet, L., Schweiser, R.C., Maikoe,T., Raaijmakers, J.A., Lammers, J.W., Koenderman, L. Comparison of the roles of mitogen-activated protein kinase and phosphatidylinositol 3-kinase signal transduction in neutrophil effector function. Biochem. J. 329: 121-130, 1998.

Coffer, P.J., Schweiser, R.C., Dubois, G.R., Maikoe,T., Lammers, J.W., Koenderman, L. Analysis of signal transduction pathways in human eosinophils activated by

chemoattractants and the T-helper 2-derived cytokines interleukin-4 and interleukin-5. Blood 91: 2547-2557, 1998a.

Colamonici, O., Yan, H., Domanski, P., Handa, R., Smalley, D., Mullersman, J., Witte, M., Krishnan, K., Krolewski, J.J. Direct binding to and tyrosine phosphorylation of the alpha subunit of the type I interferon receptor by p135tyk2 tyrosine kinase. Mol. Cell. Biol. 14: 8133-8142, 1994.

Colamonici, O.R., Uyttendaele, H., Domanski, P, Yan, H., Krolewski, J.J. p135tyk2, an interferon-α-activated tyrosine kinase, is physically associated with an Interferon-α receptor. J. Biol. Chem. 269: 3518-3522, 1994a.

Curtin, M.L. Current status of platelet-activating factor antagonists. Exp.Opin. Ther. Patents 8: 703-711, 1998.

Dagenais, P., Thivierge, M., Parent, J.-L., Stankova, J., Rola-Pleszczynski, M. Augmented expression of platelet-activating factor receptor gene by TNF-alpha through transcriptional activation in human monocytes. J. Leukocyte Biol. 61: 106-112, 1997.

Dagenais, P., Thivierge, M., Stankova, J., Rola-Pleszczynski, M. Modulation of platelet-activating factor receptor (PAFR) gene expression via NF kappa B in MonoMac-1 cells. Inflamm. Res. 46, suppl. 2: S161-S162, 1997a.

Darnell, J.E.Jr., Kerr, I.M., Stark, G.R. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264: 1415-1421, 1994.

Darnell, J.E. Jr. STATs and gene regulation Science 277: 1630-1635, 1997.

David, M., Che, H.E., Goelz, S., Larner, A.C., Neel, B.G. Differential regulation of alpha/beta interferon-stimulated Jak/STAT pathway by the SH2-domain-containing tyrosine phosphatase SHPTP1. Mol. Cell. Biol. 15: 7050-7058, 1995.

DeCoster, M.A., Mukherjee, P.K., Davis, R.J., Bazan, N.G. Platelet-activating factor is a downstream messenger of kainate-induced activation of mitogen-activated protein kinases in primary hippocampal neurons. J. Neurosci. Res. 53: 297-303, 1998.

DeFea, K.A., Vaughn, Z.D., O'Bryan, E.M., Nishijima, D., Dery, O., Bunnett, N.W. The proliferative and antiapoptotic effects of substance P are facilitated by formation of a beta -arrestin-dependent scaffolding complex. Proc. Natl. Acad. Sci. 97: 11086-11091, 2000.

Demopoulos, C.A., Pinckard, R.N., Hanahan, D.J. Platelet-activating factor. Evidence for 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine as the active component. J. Biol. Chem. 254: 9355-9358, 1979.

Deo, D. D., Axelrad, T. W., Robert, E. G., Marcheselli, V., Bazan, N. G., Hunt, J. D. Phosphorylation of STAT-3 in response to basic fibroblast growth factor occurs through a mechanism involving platelet-activating factor, JAK-2, and Src in human umbilical vein endothelial cells. Evidence for a dual kinase mechanism. J Biol. Chem. 277:21237-21245, 2002.

Dhar, A., Paul, A.K., Shukla, S.D. Platelet-activating factor stimulation of tyrosine kinase and its relationship to phospholipase C in rabbit platelets: studies with genistein and monoclonal antibody to phosphotyrosine. Mol. Pharmacol. 37: 519-525, 1990.

Dhar A. and Shukla S. D. Electrotransjection of pp60v-src monoclonal antibody inhibits activation of phospholipase C in platelets. A new mechanism for platelet-activating factor responses. J Biol Chem 269:9123, 1994.

DiSanto, J.P., Muller, W., Guy-Grand, D., Fischer, A., Rajewsky, K. Lymphoid development in mice with targeted deletion of the interleukin 2 receptor γ chain. Proc. Natl. Acad. Sci. 92: 377-381, 1995.

Duchene, J., Schanstra, J.P., Pecher, C., Pizard, A., Susini, C., Esteve, J.P., Bascands, J.L., Girolami, J.P. A novel protein-protein interaction between a G-protein coupled receptor and phosphatase SHP-2 is involved in bradykinin-induced inhibition of cell proliferation. J. Biol. Chem. 277: 40375-40383, 2002.

Duhe, R.J. and Farrar, W.L. Characterization of active and inactive forms of jak2 protein-tyrosine kinase produced via baculovirus expression vector system. J. Biol. Chem. 270: 23084-23089, 1995.

Duhe, R.J., Wang L.H., Farrar, W.L. Negative regulation of Janus kinases. Cell. Biochem. Biophys. 34: 17-59, 2001.

Endo, T.A, Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Matsumoto, A., Tanimura, S., Ohtsubo, M., Misawa, H., Miyazaki, T., Leonor, N., Taniguchi, T., Fujita, T., Kanakura, Y., Komiya, S., Yoshimura, A. A new protein containing an SH2 domain that inhibits JAK kinases. Nature 387: 921-924, 1997.

Firmbach-Kraft, I., Buers, M., Sjows, R., Dalla-Favera, R., Krolewski, J.J. Tyk2, prototype of a novel class of non-receptor tyrosine kinase genes. Oncogene 5: 1329-1336, 1990.

Feng, J., Witthuhn, B.A., Matsuda, T., Kohlhuber, F., Kerr, I.M., Ihle, J.N. Activation of Jak2 catalytic activity requires phosphorylation of Y^{1007} in the kinase activation loop. Mol. Cell Biol. 17: 2497-2501, 1997.

Fields, T.A. and Casey, P.J. Signalling functions and biochemical properties of pertussis toxin-resistant G-proteins. Biochem. J. 321: 561-571, 1997.

Frank, G.D., Motley, E.D., Sasaki, T., Ohba, M., Kuroki, T., Inagami, T., Eguchi, S. Requirement of Ca(2+) and PKCdelta for Janus kinase 2 activation by angiotensin II: involvement of Pyk2. Mol. Endocrinol. 16: 367-377, 2002.

Fujitani, Y., Hibi, M., Fukada, T., Takahashi-Tezuka, M., Yoshida, H., Yamaguchi, T., Sugiyama, K., Yamanaka, Y., Nakajima, K., Hirano, T. An alternative pathway for STAT activation that is mediated by the direct interaction between JAK and STAT. Oncogene 14:751-761, 1997.

Fukunaga, K., Ishii, S., Asano, K., Yokomizo, T., Shiomi, T., Shimizu, T., Yamaguchi, K. Single nucleotide polyporphism of human Platelet-activating factor receptor impaires G-protein activation. J. Biol. Chem. 276: 43025-43030, 2001.

Gauzzi, M.C., Velazquez, L., McKendry, R., Mogansen, K.E., Fellous, M., Pellegrini, S. Interferon-alpha-dependent activation of Tyk2 requires phosphorylation of positive regulatory tyrosines by another kinase. J. Biol. Chem. 271: 20494-20500, 1996.

Geijen, N., vanDelft, S., Raaijmakers, J.A.M., Lammers, J.W.J., Collard, J.G., Koenderman, L., Coffer, P.J. Regulation of p21rac activation in human neutrophils. Blood 94: 1121-1130, 1999.

Goldshtein J.L., Brown, M.S., Anderson, R.G., Russell, D.w., Schneider, W.J. Receptor mediated endocytosis. Annu. Rev. Cell. Biol. 1: 1-39, 1985.

Greenlund, A.C., Morales, M.O., Viviano, B.L., Yan, H., Krolewski, J.J., Schreiber, R.D. STAT recruitment by tyrosine-phosphorylated cytokine receptors: and ordered reversible affinity-driven process. Immunity 2: 677-687, 1995.

Guillet-Deniau, I., Burnol, A.-F., Girard, J. Identification and localization of a skeletal Muscle Serotonin 5-HT2A receptor coupled to the Jak/STAT pathway. J. Biol.Chem. 272: 14825-14829, 1997.

Hamm, H.E. The many faces of G protein signaling. J. Biol. Chem. 273: 669-672, 1998.

Haspel R.L., Salditt-Georgieff, M., Darnell, J.E. The rapid inactivation of nuclear tyrosine phosphorylated Stat1 depends upon a protein tyrosine phosphatase. EMBO J. 15: 6262-6268, 1996.

Harpur, A.G., Andres, A.C., Ziemiecki, A., Aston, R.R., Wilks, A.F. JAK2, a third member of the JAK family of protein tyrosine kinases. Oncogene 7: 1347-1353, 1992.

Herrington, J., Rui, R., Luo, G., Yu-Lee, L., and Carter-Su, C. A functional DNA binding domain is required for growth hormone-induced nuclear accumulation of Stat5B. J. Biol. Chem. 274: 5138-5145, 1999.

Hilton, D.J., Richardson, R.T., Alexander, W.S., Viney, E.M., Willson, T.A., Sprigg, N.S., Starr, R., Nicholson, S.E., Metcalf, D., Nicola, N.A.. Twenty proteins containing a C-terminal SOCS box form five structural classes Proc. Natl. Acad. Sci. USA 95: 114-119, 1998.

Ho, Y.S., Lee, W.M., Snyderman, R. Chemoattractant-induced activation of c-fos gene expression in human monocytes. J. Exp. Med. 165: 1524-1538, 1987

Honda, Z., Nakamura, M., Miki, I., Minami, M., Wantanabe, T., Seyama, Y., Okado, H., Toh, H., Ito, K., Myamoto, T., Shimizu, T. Cloning by functional expression of platelet-activating factor from guinea pig lung. Nature 349: 342-346, 1991.

Honda, Z., Takano, T., Gotoh, Y., Nishida, E., Ito, K., Shimizu, T. Transfected platelet-activating factor receptor activates mitogen-activated protein (MAP) kinase and MAP kinase kinase in Chinese hamster ovary cells. J. Biol. Chem. 269: 2307-2315, 1994.

Hong, J.U., Venema, V.J., Liang, H., Harris, M.B., Zou, R., Venema, R.C. bradykinin activates Janus-activated kinase/signal transducers and activators of transcription (Jak/STAT) pathway in vascular endothelial cells: localization of Jak/STAT signaling proteins in plasmalemmal caveolae. Biochem. J. 351: 257-264, 2000.

Hunt, R. A., Bhat, G. J., Baker, K. M. Angiotensin II–Stimulated Induction of *sis*-Inducing Factor Is Mediated by Pertussis Toxin–Insensitive G_q Proteins in Cardiac Myocytes. Hypertension 34: 603-608, 1999.

Hwang, S.B. Specific receptors of platelet-activating factor, receptor heterogeneity and signal transduction mechanisms. J. Lipid Mediators 2: 123-158, 1990.

Huang, S.J., Monk, P.N., Downes, C.P., Whetton, A.D. Platelet-activating factor-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate stimulates the production of reactive oxygen intermediates in macrophages. Biochem. J. 249: 839-845, 1988.

Iseki, M., Takaki, S., Takatsu, K. Molecular cloning of the mouse APS as a member of the Lnk family adaptor proteins. Biochem. Biophys. Res. Commun. 272: 45-54, 2000.

Ishii, S., Nakamura, M., Waga, I., Kume, K., Izumi, T., Shimizu, T. Cloning and characterization of a murine platelet-activating factor receptor gene. Adv. Exp. Med. Biol. 407: 347-355, 1997.

Ishii, I., Izumi, T., Tsukamoto, H., Umeyama, H., Ui, M., Shimizu, T. Alanine exchange of polar amino acids in the transmembrane domains of a Platelet-activating factor receptor generates both constitutively active and inactive mutants. J. Biol. Chem. 272: 7846-7854, 1997.

Ishii, S. and Shimizu, T. Platelet-activating factor (PAF) receptor and genetically engineered PAF receptor mutant mice. Progress in Lipid Res. 39: 41-82, 2000.

Ishii, S., Nagase, T., Tashiro, F., Ikuta, K., Sato, S., Waga, I., Kume, K., Miyazaki, J., Shimizu, T. Bronchial hyperreactivity, increased endotoxin lethality and melanocytic tumorogenesis in transgenic mice overexpressing platelet-activating factor receptor. EMBO J. 16: 133-142, 1997

Ishii, S., Kuwaki, T., Nagase, T., Maki, K., Tashiro, F., Sunaga, S., Cao, W.H., Kume, K., Fukuchi, Y., Ikuta, K., Miyazaki, J., Kumada, M., Shimizu, T. Impaired anaphylactic responses with intact sensitivity to endotoxin in mice lacking a platelet-activating factor receptor. J. Exp. Med. 187: 1779-1788, 1998.

Izumi, T., Shimizu, T. Platelet-activating factor receptor: gene expression and signal transduction. Biochim. Biophys. Acta 1259: 317-333, 1995.

Izumi, T., Takano, T., Bito, H., Nakamura, M., Mutoh, H., Honda, Z., Shimizu, T. Platelet-activating factor receptor. J. Lipid Mediators Cell Signaling 12: 429-442, 1995.

Jiao, H., Berrada, K., Yang, W., Tabrizi, M., Platinias, L.C., Yi, T. Direct association with and dephosphorylation of Jak2 kinase by the SH2-domain-containing protein tyrosine phosphatase SHP-1. Mol. Cell. Biol. 16: 6985-6992. 1996.

John, S., Robbins, C.M., Leonard, W.J. An IL-2 response element in the human IL-2 receptor alpha chain promoter is a composite element that binds STAT5, Elf-1, HMG-1(Y) and a GATA family protein. EMBO J. 15:5627-5635, 1996.

Johnstone, J.A., Kawamura, M., Kirken, R.A., Chen, Y.Q., Blake, T.B., Shibuya, K., Ortaldo, J.R., McVicar, D.W., O'Shea, J.J. Phosphorylation and activation of the Jak-3 Janus kinase in response to interleukin-2. Nature 370: 151-153, 1994.

Ju, H., Venema, V.J., Liang, H., Harris, M.B., Zou, R., Venema, R.C. Bradykinin activates the Janus-activated kinase/signal transducers and activators of transcription (JAK/STAT) pathway in vascular endothelial cells: localization of JAK/STAT signaling proteins in plasmalemmal caveolae. Biochem. J. 351: 257-264, 2000.

Kawamura, M., McVicar, D.W., Johnstone, J.A., Blake, T.B., Chen, Y.Q., Lal, B.K., Kelvin, D.J., Staples, J.E., Ortaldo, J.R., O'Shea, J.J. Molecular cloning of L-JAK, a Janus family protein-tyrosine kinase expressed in natural killer cells and activated leukocytes. Proc. Natl. Acad. Sci. USA 91: 6374-6378, 1994.

Kharagiosoff, M., Neubauer, H., Lassnig, C., Kovarik, P., Schindlier, H., Pircher, H., McCoy, B et al. Partial impairment of cytokine responses in Tyk2-deficient mice. Immunity 13: 549-560, 2000.

Klingmuller, U., Lorenz, U., Cantley, L.C., Neel, B.G., Lodish, H.F. Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. Cell 80: 729-738, 1995.

Krishnan, K., Pine, R., Krolewski, J.J. Kinase-deficient forms of Jak1 and Tyk2 inhibit interferon _ signaling in a dominant negative manner. Eur. J. Biochem. 247: 298-305, 1997.

Krolewski, J.J., Lee, R., Eddy, R., Shows, T.B., Dalla-Favera, R. Identification and chromosomal mapping of new human tyrosine kinase genes. Oncogene 5: 277-282, 1990.

Kunz, D., Gerard, N.P., Gerard, C. The human leukocyte platelet-activating factor receptor. CDNA cloning, cell surface expression and construction of a novel epitope-bearing analog. J. Biol. Chem. 267: 9101-9106, 1992.

Kuruvilla, A., Pielop, C., Shearer, W.T. Platelet-activating factor induces the tyrosine phosphorylation and activation of phospholipase C-gamma 1, Fyn and Lyn kinases, and phosphatidylinositol 3-kinase in a human B cell line. J. Immunol. 153: 5433-5442, 1994.

Lai, K.S., Jin, Y., Graham, D.K., Witthuhn, B.A., Ihle, J.N., Liu, E.T. A kinase-deficient splice variant of the human JAK3 is expressed in hematopoietic and epithelial cancer cells. J. Biol. Chem. 270: 25028-25036, 1995.

Le Gouill, Parent, J.-L., C., Rola-pleszczynski, M., Stankova, J. Role of the Cys^{90}, Cys^{95} and Cys^{173} residues in the structure and function of the human platelet-activating factor receptor. FEBS Lett. 402: 203-208, 1997.

Le Gouill, C., Parent, J.-L., Caron, C.-A., Gaudreau, R., Volkov, L., Rola-Pleszczynski, M., Stankova, J. Selective modulation of wild type receptor functions by mutants of G-protein-coupled receptors. J. Biol. Chem. 274: 12548-12554, 1999.

Lin, J.X., Migone, T.S., Tsang, M., Friedmann, M., Weatherbee, J.A., Zhou, L., Yamauchi, A., Bloom, E.T., Mietz, J., John, S., Leonard, W.J. The role of shared receptor motifs and common STAT proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13 and IL-15. Immunity 2: 331-339, 1995.

Liu, K.D., Gaffe, S.L., Goldsmith, M.A. Jak/STAT signaling by cytokine receptors. Curr. Opin. Immunol. 10: 271-278, 1998.

Liu, B., Nakashima, S., Kanoh, H., Takano, T., Shimizu, T., Nozawa, Y. Activation of phospholipase D in Chinese hamster ovary cells expressing platelet-activating factor receptor. J. Biochem 16: 882-891, 1994.

Livnah, O., Stura, E.A., Middleton, S.A., Johnson, D.L., Joliffe, L.K., Wilson, I.A. Crystallographic evidence for preformed dimers of erythropoietin receptor before ligand activation. Science 283: 987-990, 1999.

Look, D.C., Pelletier, M.R., Tidwell, R.M., Roswit, W.T., Holtzman, M.J. STAT1 depends on transcriptional synergy with Sp1. J. Biol. Chem. 27: 30264-30267, 1995.

Losman J.A., Chen, X.P., Hilton, D., Rothman, P. Cutting edge: SOCS-1 is a potent inhibitor of IL-4 signal transduction. J. Immunol. 162: 3770-3774, 1999.

Lukashova, V., Lukashev, V.A., Lukashev, V.V., Rola-Pleszczysnki, M., Stankova, J. Theoretical model of interaction: Platelet-activating factor receptor and tyrosine kinase tyk2. Bioinformatics in genome regulation and structure. 204-206, 2000.

Lukashova, V., Asselin, C., Krolewski, J.J., Rola-Pleszczynski, M., Stankova, J.. G-protein independent activation of Tyk2 by the Platelet-Activating Factor receptor. J. Biol. Chem. 276: 24113-24121, 2001.

Luttrell, L.M., Ferguson, S.S., Daaka, Y., Miller, W.E., Maudsley, S., Della Rocca, G.J., Lin, F., Kawakatsu, H., Owada, K., Luttrell, D.K., Caron, M.G., Lefkowitz, R.J. Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. Science 283: 655-661, 1999.

Macchi, P., Villa, A., Giliani, S., Sacco, M.G., Frattini, A., Porta, F. et al. Mutations of Jak3 gene in patients with autosomal severe combined immune deficiency (SCID). Nature 377: 65-68, 1995.

Marrero, M.B., Schieffer, B., Paxton, W.G., Heerdt, L., Berk, B.C., Delafontaine, P., Bernstein, K.E. Direct stimulation of Jak/STAT pathway by the angiotensin II AT1 receptor. Nature 375: 247-250, 1995.

Marrero, M.B., Venema, V.J., Ju, H., Eaton, D.C., Venema, R.C. Regulation of angiotensin II-stimulated Jak2 tyrosine phosphorylation: roles of SHP-1 and SHP-2. Am. J. Physiol. 275: C1216-1222, 1998.

Masuhara, M., Sakamoto, H., Matsumoto, A., Suzuki, R., Yasukawa, H., Mitsui, K., Wakioka, T., Tanimura, S., Sasaki, A., Misawa, H., Yokouchi, M., Ohtsubo, M., Yoshimura, A. Cloning and characterization of novel CIS family gene. Biochem. Biophys. Res. Commun. 239: 439-446, 1997.

Matuoka, K., Miki, H., Takahashi, K., Takenawa, T. A novel ligand for an SH3 domain of the adaptor protein Nck bears an SH2 domain and nuclear signaling motifs. Biochem. Biophys. Res. Commun. 239: 488-492, 1997.

Matsumoto, A., Masuhara, M., Mitsui, K., Yokouchi, M., Ohtsubo, M., Misawa, H., Miyajima, A., Yoshimura, A. CIS, a cytokine inducible SH2 protein, is a target of the JAK-STAT5 pathway and modulates STAT5 activation. Blood 89: 3148-3154, 1997.

Mazer, B., Domenico, J., Sawami, H., Gelfand, E.W. Platelet-activating factor induces an increase in intracellular calcium and expression of regulatory genes in human B lymphoblastoid cells. J. Immunol. 146: 1914-1920, 1991.

McBride, K.M., McDonald, C., Reich, N.C. Nuclear export signal located within the DNA-binding domain of the STAT1 transcription factor. EMBO J. 19: 6196-6206, 2000.

McBride, K.M., Banninger, G., McDonald, C., Reich, N.C. Regulated nuclear import of the STAT1 transcription factor by direct binding of importin-α. EMBO J. 21: 1754-1763, 2002.

McDonald, P.H., Chow, C.-W., Miller, W.E., Laporte, S.A., Field, M.E., Lin, F.-T., Davis, R.J., Lefkowitz, R.J. Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. Science 290: 1574-1577, 2000.

Melen, K., Kinnunen, L., Julkunen, I. Arginine/lysine-rich structural element is involved in interferon-induced nuclear import of STATs. J. Biol. Chem. 276: 16447-16455, 2001.

Mellado, M., Rodriguez-Frade, J.M., Aragay, G.del Real., Martin, A.M., Vila-Coro, A.G., Serrano, A., Mayor, F., Martinez-A., Jr. and C. The Chemokine Monocyte Protein

1 triggers Janus kinase 2 activation and tyrosine phosphorylation of the CCR2B receptor. J. Imunol. 161: 805-813, 1998.

Metcalf, D., Greenhalgh, C.J., Viney, E., Willson, T.A., Starr, R., Nicola, N.A., Hilton, D.J., Alexander, W.S. Gigantism in mice lacking suppressor of cytokine signaling-2. Nature 405: 1069-1073, 2000

Meyer, T., Begitt, A., Lodige, I., van, Rossum, M., Vinkemeier, U., Constitutive and IFN-γ-induced nuclear import of STAT1 proceed through independent pathways. EMBO J. 21: 344-354, 2002.

Mikita, T., Campbell, D., Wu, P., Williamson, K., Shindler,U. Requirements for interleukin-4-induced gene expression and functional characterization of STAT6. Mol. Cell. Biol. 16: 5811-5820, 1996.

M'Rabet, L., Coffer, P., Zwartkruis, F., Franke, B., Segal, A.W., Koenderman, L., Bos, J.L. Activation of the small GTPase rap1 in human neutrophils. Blood 92: 2133-2140, 1998.

M'Rabet, L., Coffer, P.J., Wolthuis, R.M.F., Zwartkruis, F., Koenderman, L., Bos, J.L. Differential fMet-Leu-Phe- and platelet-activating factor-induced signaling toward Ral activation in primary human neutrophils. J. Biol. Chem. 274: 21847-21852, 1999.

Mui, A.L., Wakao, H., Harda, N., O'Farrel, A., Miyajima, A. Interleukin-3, granulocye-macrophage colony-stimulaing factor, and interleukin-5 transduce signals through two forms of STAT5. J. Leukocyte Biol. 57: 799-803, 1995.

Muller, M., Briscoe, J., Laxton, C., Gushin, D., Ziemiecki, A. Silvennoinen, O., Harpur, A.G., Barbieri, G., Witthuhn, B.A., Schindler, C., et al. The protein tyrosine kinase JAK1 complements defects in interferon-alpha/beta and -gamma signal transduction. Nature 366: 129-135, 1993.

Mutoh, H., Bito, H., Minami, M., Nakamura, M., Honda, Z., Izumi, T., Nakata, R., Kurachi, Y., Terano, A., Shimizu, T. FEBS Lett. 322: 129-134, 1993.

Mutoh, H., Ishii, S., Izumi, T., Kato, S., Shimizu, T. Platelet-activating factor (PAF) positively auto-regulates the expression of human PAF receptor transcript 1 (leukocyte-type) through NF-kappa B. Biochem. Biophys. Res. Commun. 205: 1137-1142, 1994.

Mutoh, H., Kume, K., Sato, S., Kato, S., Shimizu, T. Positive and negative regulations of human platelet-activating factor receptor transcript 2 (tissue-type) by estrogen and TGF-beta 1. Biochem.Biophys. Res. Commun. 205: 1130-1136, 1994a.

Mutoh, H., Fukuda, T., Masushige, S., Sasaki, H., Shimizu, T., Kato, S. Tissue-specific response of the human platelet-activating factor receptor gene to retinoic acid and thyroid hormone by alternative promoter usage. Proc. Natl. Acad. Sci. USA 93: 774-780, 1996

Naccache, P.H., Molski, M.M., Volpi, M., Becker, E.L., Sha'afi, R.I. Unique inhibitory profile of platelet activating factor induced calcium mobilization, polyphosphoinositide turnover and granule enzyme secretion in rabbit neutrophils towards pertussis toxin and phorbol ester. Biochem. Biophys. Res.Commun. 130: 677-684, 1985.

Nagase, T., Ishii, S., Kume, K., Uozumi, N., Izumi, T., Ouchi Y, Shimizu T. Platelet-activating factor mediates acid-induced lung injury in genetically engineered mice. J. Clin. Invest. 104: 1071-1076, 1999.

Naka, T., Narazaki, M., Hirata, M., Matsumoto, T., Minamoto, S., Aono, A., Nishimoto, N., Kajita, T., Taga, T., Yoshizaki, K., Akira, S., Kishimoto, T. Structure and function of a new STAT-induced STAT inhibitor. Nature 387: 924-928, 1997.

Naka, T., Tsutsui, H., Fujimoto, M., Kawazoe, Y., Kohzaki, H., Morita, Y., Nakagawa, R., Narazaki, M., Adachi, K., Yoshimoto, T. SOSC-1/SSI/1-deficient NKT cells participate in severe hepatitis through disregulated cross-talk inhibition of IFN-γ and IL-4 signaling in vivo. Immunity 14: 535-545, 2001.

Nakamura, N., Chin, H., Miyasaka, N., Miura, O. An epidermal growth factor receptor/Jak2 tyrosine kinase domain chimera induces tyrosine phosphorylation of Stat5 and transduces a growth signal in hematopoietic cells. J. Biol. Chem. 271: 19483-19488, 1996.

Nakamura, M., Honda, Z., Izumi, T., Sakanaka, C., Mutoh, H., Minami, M., Bito, H., Seyama, Y., Matsumoto, T., Noma, M., Shimizu, T. Molecular cloning and expression of platelet-activating factor receptor from human leukocytes. J. Biol. Chem. 266: 20400-20405, 1991

Neubauer, H., Cumano, A., Muller, M., Wu, H., Huffstadtm U., Pfeffer, K. Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. Cell 93: 397-409, 1998.

Nurnberg, B. and Ahnert-Hilber, G. Potential toles of heterotrimeric G proteins of the endomembrane system. FEBS Lett. 389: 61-65, 1996.

O'Brien, K.B., O'Shea, J.J., Carter-Su, C. SH2-B family members differentially regulate Jak family tyrosine kinases. J. Biol. Chem. 277: 8673-8681, 2002.

Ohya, K, Kajigaya, S., Yamashita, Y., Miyazato, A., Hatake, K., Miura, Y., Ikeda, U., Shimada, K., Ozawa, K., Mano, H. SOCS-1/JAB/SSI-1 can bind to and suppress Tec protein-tyrosine kinase. J. Biol. Chem. 272: 27178-27182, 1997.

Ouellet, S., Muller, E., Rola-Pleszczynski, M. IFN-gamma up-regulates platelet-activating factor receptor gene expression in human monocytes. J. Immunol. 152: 5092-5099, 1994.

Pang, J.-H., Hung, R.-Y., Wu, C.-J., Fang, Y.-Y., Chau, L.-Y. Functional characterization of the promoter region of the platelet-activating factor receptor gene. Identification of an initiator element essential for gene expression in myeloid cells. J. Biol. Chem. 270: 14123-14129, 1995.

Parent, J.-L., Le Gouill, C., Escher, E., Rola-Pleszczynzki, M., Stankova, J. Identification of transmembrane domain residues determinant in the structure-function relationship of the human Platelet-activating factor receptor by site-directed mutagenesis. J. Biol. Chem. 271: 23298-23303, 1996.

Parent, J.-L. and Stankova, J. Transforming growth factor-beta up-regulates the gene expression of the human platelet-activating factor receptor in monocytic and B cell lines. Biochem. Biophys. Res. Commun. 197: 1443-1449, 1993.

Parent, J.-L., Le Gouill, C., de Brum-Fernandes, A., Rola-Pleszczynski, M., Stankova, J. Mutations of two adjacent amino acids generate inactive and constitutively active forms of the human platelet-activating factor receptor. J. Biol. Chem. 271: 7949-7955, 1996.

Parent, J.-L., Le Gouill, C., Escher, E., A., Rola-Pleszczynski, M., Stankova, J. Identification of transmembrane domain residues determinant in the structure-function relationship of the human platelet-activating factor receptor by site-directed mutagenesis. J. Biol. Chem. 271: 23298-23303, 1996.

Parent, J.-L., Le Gouill, C., Rola-Pleszczynski, M., Stankova, J. Mutation of an aspartate at position 63 in the human platelet-activating factor receptor augments binding affinity but abolishes G-protein-coupling and inositol phosphate production. Biochem. Biophys. Res. Commun. 219: 968-975, 1996.

Parganas, E., Wang, D., Stravopodis, D., Topham, D.J., Marine, J.C., Teglund, S., et al. Jak2 is essential for signaling through a variety of cytokine receptors. Cell 93: 385-395, 1998.

Pierce, K.L., Luttrell, L.M., Lefkowitz, R.J. New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. Oncogene 20: 1532-1539, 2001

Pellegrine, S. and Dusanter-Fourt, I. The structure, regulation and function of the Janus kinmases (Jaks) and the signal transducers and activators of transcription (STATs). Eur. J. Biochem. 248: 615-633, 1997.

Peplow, P.V. Regulation of platelet-activating factor (PAF) activity in human diseases by phospholipase A2 inhibitors, PAF acetylhydrolases, PAF receptor antagonists and free radical scavengers. Prostaglandins Leukot Essent Fatty Acids. 61: 65-82, 1999.

Premzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., Ullrich, A. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature 402: 884-888, 1999.

Qureshi, S.A., Leung, S., Kerr, I.M., Stark, G.R., Darnell, J.E.Jr Function of STAT2 protein in transcriptional activation by alpha interferon. Mol. Cell. Biol. 16: 288-293, 1996.

Rane, S.G. and Reddy, E.P. JAK3: a novel JAK kinase associated with terminal differentiation of hematopoietic cells. Oncogene 9: 2415-2423, 1994.

Rane, S.G. and Reddy, E.P. Janus kinases: components of multiple signaling pathways. Oncogene 19: 5662-5679, 2000.

Rémy, I., Wilson, I.A., Michnik, S.W., Erythropoietin receptor activation by a ligand-induced conformational change. Science 283: 990-993, 1999.

Rezaul, K., Sada, K., Inazu, T., Yammura, H. Platelet-Activating Factor stimulates calcium-dependent activation of protein tyrosine kinase Syk in a human B cell line. Biochem Biophys. Res. Commun. 239: 23-27, 1997.

Roberts, A.W., Robb, L., Rakar, S., Hartley, L., Cluse, L., Nicola, N.A., Metcalf, D., Hilton, D.J., Alexander, W.S. Placental defects and ebryonic lethality in mice lacking suppressor of cytokine signaling 3. Proc. Natl. Acad. Sci. USA 98 : 9324-9329, 2001.

Rodig, S.J., Meraz, M.A., White, J.M., Lampe, P.A., Riley, J.K., Arthur, C.D., et al. Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. Cell 93: 373-383, 1998.

Rodriguez, C.G., Cundell, D.R., Tuomanen, D.I., Kolakowski, L.F. Jr., Gerard, C., Gerard, N.P. The role of N-glycosylation for functional expression of the human Platelet-activating factor receptor. J. Biol. Chem. 270: 25178-25184, 1995.

Rola-Pleszczynski, M., Thivierge, M., Gagnon, N., Lacasse, C., Stankova, J. Differential regulation of cytokine and cytokine receptor genes by PAF, LTB4 and PGE2. J. Lipid Mediators 6 :175-181, 1993.

Russel, S.M., Tayebi, N., N., Nakajima, H., Riedy, M.C., Roberts, J.L., Aman, M.J., Mutation of Jak3 gene in a patient with SCID: essential role of Jak3 in lymphoid development. Science 270: 797-800, 1995

Saharinen, P., Takaluoma, K., Silvennoinen, O. Regulation of the Jak2 tyrosine kinase by its pseudokinase domain. Mol. Cell. Biol. 20: 3387-3395, 2000.

Sakai, I., Nabell, L., Kraft, A.S. Signal transduction by a CD16/CD7/Jak2 fusion protein. J. Biol. Chem. 271: 23634-23637, 1995.

Sayeski, P.P., Ali, M.S., Frank, S.J., Bernstein, K.E. The angiotensin II-dependent nuclear translocation of STAT1 is mediated by the Jak2 protein motif ^{231}YRFRR. J. Biol. Chem. 276: 10556-10563, 2001.

Sayeski, P. P., Ali, M. S., Hawks, K., Frank, S. J K., Bernstein. E. 1999. The angiotensin II-dependent association of Jak2 and c-Src requires the N-terminus of Jak2 and the SH2 domain of c-Src. Circ Res 84:1332-1338, 1999.

Schindler, C. and Darnell, J.E.Jr. Transcriptional responses to polypeptide ligands: the Jak/STAT pathway. Annu. Rev. Biochem. 64: 621-651, 1995.

Shi, L.C., Wang, H.Y., Horwitz, J., Friedman, E. Guanine nucleotide regulatory proteins, Gq and Gi1/2, mediate platelet-activating factor-stimulated phosphoinositide metabolism in immortalized hippocampal cells. J. Neurochem. 67: 1478-1484, 1996.

Shimizu, T., Mutoh, H., Kato, S. Platelet-activating factor receptor. Gene structure and tissue-specific regulation. Platelet-activating factor and related lipid mediators. 2nd plenum press, New York, 79-84, 1996.

Shimizu, T and Mutoh, H. Structure and regulation of platelet activating factor receptor gene. Adv. Exp. Med. Biol. 407: 197-204, 1997.

Shimoda, K., Kato, K., Aoki, K., Matsuda, T., Miyamoto, A., Shibamori, M., Yamashita, M et al. Tyk2 plays a restricted role in IFNα signaling, although it is required for IL-12-mediated T cell function. Immunity 13: 561-571, 2000.

Shirasaki, H., Nishikawa M., Adcock, I.M., Mak, J.C.W., Sakamoto, T., Shimizu, T., Barnes, P.J. Expression of platelet-activating factor receptor mRNA in human and guinea pig lung. Am. J. Respir. Cell Mol. Biol. 10: 533-537, 1994.

Shuai, K., Stark, G.R., Kerr, I.M., Darnell, J.E.Jr. A single phosphotyrosine residue of STAT91 required for gene activation by interferon-gamma. Science 261: 1744-1746, 1993.

Shukla, S.D. Inositol phospholipid turnover in PAF transmembrane singling. Lipids 26: 1028-1033, 1991.

Shultz, L.D., Rajan, T.V., Greiner, D.L. Severe defects in immunity and hematopoiesis caused by SHP-1 protein-tyrosine-phosphatase deficiency. Trend Biotechnol. 15: 302-307, 1997.

Shultz L.D., Schweitzer, P.A., Rajan, T.V., Yi, T., Ihle, J.N., Matthews, R.J., Thomas, M.L., Beier, D.R.. Mutations at the murine motheaten locus are within the hematopoietic cell protein-tyrosine phosphatase (Hcph) gene. Cell 73: 1445-1454, 1993

Snyder, F., Fitzgerald, V., Blank M.L Platelet-activating factor and related lipid mediators. Advances in Experimental Medicine and Biology, Plenum Press, 416: 5-16, 1996.

Soldi, R., Sanavio, F., Aglietta, M., Primo, L., Defilippi, P., Marchisio, P.C., Bussolino, F. Platelet-activating factor (PAF) induces the early tyrosine phosphorylation of focal adhesion kinase (p125FAK) in human endothelial cells. Oncogene 13: 515-525, 1996

Starr R., Willson, T.A., Viney, E.M., Murray, L.J., Rayner, J.R., Jenkins, B.J., Gonda, T.J., Alexander, W.S., Metcalf, D., Nicola, N.A., Hilton, D.J. A family of cytokine-inducible inhibitors of signaling. Nature 387: 917-921, 1997.

Starr R. and Hilton D.J. Negative regulation of the JAK/STAT pathway. BioEssays, 21: 47-52, 1999.

Stocklin, E., Wissler, M., Gouilleux, F., Groner, B. Functional interactions between STAT5 and glucocorticoid receptor. Nature 383: 726-728, 1996.

Symes A.,. Stahl N., Reeves, S.A., Farruggella, T., Servidei, T., Gearan, T., Yancopoulos, G., Fink, J.S. The protein tyrosine phosphatase SHP-2 negatively regulates ciliary neurotrophic factor induction of gene expression. Curr. Biol. 7: 697-700, 1997.

Takeda, K., Akira, S. STAT family of transcription factors in cytokine-mediated biological responses. Cytokine growth factor rev. 11:199-207, 2000.

Takahashi, T. and Shirasawa, T. Molecular cloning of rat JAK3, a novel member of the JAK family of protein tyrosine kinases. FEBS Lett. 342: 1240128, 1994.

Tanner, J.W., Chen, W., Young, R.L., Longmore, G.D., Shaw, A.S. The conserved box 1 motif of cytokine receptors is required for association with JAK kinases. J. Biol. Chem. 270: 6523-6530, 1995.

Terashita, Z., Tsushima, S., Yoshioka, Y., Nomura, H., Inada, Y., Nishikawa, K. CV-3988 - a specific antagonist of platelet activating factor (PAF). Life Sci. 32:1975-82, 1983.

Thivierge, M., Alami, N., Muller, E., de Brum Fernandes, F., Rola-Pleszczysnki, M. Transcriptional modulation of platelet-activating factor receptor gene expression by cyclic AMP. J. Biol. Chem. 268: 17457-1717462, 1993.

Thivierge, M., Parent, J.-L., Stankova, J., Rola-Pleszcsynski, M. Modulation of human platelet-activating factor receptor gene expression by protein kinase C activation. J. Immunol. 157: 4681-4687, 1996.

Tkano, T., Honda, Z.-I., Sakanaka, C., Izumi, T., Kameyama, K., Haga, K., Haga, T., Kurokawa, K., Shimizu, T. Role of cytoplasmic tail phosphorylation sites of Platelet-activating factor receptor in agonist-induced desensitization. J. Biol. Chem. 269: 22453-22458, 1994.

Tojo, A., Kasuga, M., Urabe, A., Takaku, F. Vanadate can replace interleukin 3 for transient growth of factor-dependent cells. Exp. Cell Res. 171: 16-23, 1987.

Tripathi, Y.B., Kandala, J.C., Guntaka, R.V., Lim, R.W., Shukla, S.D. Platelet activating factor induces expression of early response genes c-fos and TIS-1 in human epidermoid carcinoma A-431 cells. Life Sci. 49: 1761-1767, 1991.

Tsui, H.W., Siminovitch, K.A., de Souza, L., Tsui, F.W. Motheaten and viable motheaten mice have mutations in the haematopoietic cell phosphatase gene. Nature Genet. 4: 124-129, 1993.

Ungureanu, D., Saharinen, P., Junttila, I., Hilton, D.J., Silnennoinen, O. Regulation of Jak2 through the Ubiquitin-proteasome pathway involves phosphorylation of Jak2 on Y1007 and interaction with SOCS-1. Mol. Cell. Biol. 22: 3316-3326, 2002

Van Biezen, Hawes, B.E., Raymond, J.R., Luttrell, L.M., Koch, W.J., Lefkowitz, R.J. G(o)-protein alpha-subunits activate mitogen-activated protein kinase via a novel protein kinase C-dependent mechanism. J. Biol. Chem. 271: 1266-1269, 1996.

VanderKuur, J., Allevato, G., Billestrup, N., Norsdedt, G., Carter-Su, C. Growth hormone-promoted tyrosyl phosphorylation of SHC proteins and SCH association with Grb2. J. Biol. Chem. 270: 7587-7593, 1995.

Van Koppen, C.J., Lenz, W., Nunes, J.P., Zhang, C., Schmidt, M., Jakobs, K.H. The role of membrane proximal threonine residues conserved among guanine-nucleotide-binding-protein-coupled receptors in internalization of the m4 muscarinic acetylcholine receptor. Eur. J. Biochem. 234: 536-541, 1995.

Velazquez, L., Fellous, M., Stark, G.R., Pellegrini, S. A protein tyrosine kinase in the interferon alpha/beta signaling pathway. Cell 70: 313-322, 1992.

Velazquez, L., Mogensen, K.E., Barbieri, G., Fellous, M., Uze, G., Pellegrini, S. Distinct domains of the protein tyrosine kinase Tyk2 required for binding of Interferon-α/β and for signal transduction. J. Biol. Chem. 270: 3327-3334, 1995.

Verbsky, J.W., Bach, E.A., Fang, Y.F., Yang, L., Randolph, D.A., Fields, L.E. J. Biol. Chem. 271: 13976-13980, 1996.

Vila-Coro, A.J., Rodriguez-Frade, J.M., Martin de Ana, Moreno-Ortiz, M.C., Martinez.-A., C., Mellado, M. The chemokine SD1-α triggers CXCR4 receptor dimerization and activates the Jak/STAT pathway. FASEB J. 13: 1699-1710, 1999.

Wakioka, T., Sasaki, A., Mitsui, K., Yokouchi, M., Inoue, A., Komiya, S., Yoshimura, A. Leukemia 13: 760-767, 1999.

Wang, D., Stravopodis, D., Teglund, S., Kitasawa, J., Uhle, J.N. Naturally occuring dominant negative variants of STAT5. Mol. Cell. Biol. 16: 6141-6148, 1996.

Wang, Y.D., Wong, K., Wood, W.I. Intracellular tyrosine residues of the human growth hormone receptor are not required for the signalling of proliferation or Jak/STAT activation. J. Biol. Chem. 270: 7021-7024, 1995.

Watanabe, S., Arai, K.-I. Roles of the Jak-STAT system in signal transduction via cytokine receptors. Curr. Opin Gen. Dev. 6: 587-596, 1996.

Watling, D., Gushin, D., Muller, M., Silvennoinen, O., Witthuhnn, B.A., Quelle, F.W., Rogers, N.C., Schindler, C., Stark, G.R., Ihle, J.N., et al. Complementation by the protein tyrosine kinase JAK2 of a mutant cell line defective in the interferon-gamma signal transduction pathway. Nature 366: 166-170, 1993.

Wilks, A.F. Two putative protein-tyrosine kinases identified by application of the polymerase chain reaction. Proc. Nalt. Acad. Sci. USA 86: 1603-1607, 1989.

Wilks, A.F. Cloning members of protein-tyrosine kinase family using polymerase chain reaction. Methods Enzymol. 200: 533-546, 1991

Winston, L.A and Hunter T. Jak2, Ras and Raf are required for activation of extracellular signal-regulated kinase/mitogen-activated protein kinase by growth hormone. J. Biol. Chem. 270: 30837-30840, 1995.

Witthuhn, B.A., Quelle, F.W., Silvennoinen, O., Yi, T., Tang, B., Miura, O., Ihle, J.N. Jak2 associates with the erythropoeitin receptor and is tyrosine phosphorylated and activated following EPO stimulation. Cell 74: 227-236, 1993

Witthuhn, B.A., Silvennoinen, O., Miura, O., Lai, K.S., Cwik, C., Liu, E.T., Ihle, J.N. Involvement of the Jak-3 Janus kinase in signaling by interleukins 2 and 4 in lymphoid and myeloid cells. Nature 370: 1530157, 1994.

Witthuhn, B.A., Williams, M.D., Kerawalla, H., Uckin, F.M. Differential substrate recognition capabilities of Janus family protein kinases within the interleukin-2 receptor (IL-2R) system: Jak3 as a potential molecular target for treatment of leukemia with a hyperactive Jak/STAT signaling machinery. Leuk. Lymphoma 32: 289-297, 1999.

Wong, M., Uddin, S., Majchrzak, B., Huynh, T., Proudfoot, A.E.I., Platanias, L.C., Fish, E.N. RANTES activates Jak2 and Jak3 to regulate engagement of multiple signaling pathways in T cells. J. Biol. Chem. 276: 11427-11431, 2001

Yan, H., Piazza, F., Krishnan, K., Pine, R., Krolewski, J.J. Definition of the Interferon-α receptor-binding domain on the Tyk2 kinase. J. Biol. Chem. 273: 4046-4051, 1998

Yang, H.H., Pang, J.H., Hung, R.Y., Chau, L.Y. Transcriptional regulation of platelet-activating factor receptor gene in B lymphoblastoid Ramos cells by TGF-beta. J. Immunol. 158: 2771-2778, 1997.

Ye, R.D., Kravchenko, V.V., Pan, Z., Feng, L. Stimulation of NF-κB activation and gene expression by platelet-activating factor receptor. Platelet activating factor and related lipid mediators 2. Plenum Press, New York, 1996.

Yeh, T.C., Dondi, E., Uze, G., Pellegrini, S. A dual role for the kinase-like domain of the tyrosine kinase Tyk2 in interferon-α signaling. Proc. Natl, Acad. Sci. 97: 8991-8996, 2000.

Yetter, A., Uddin, S., Krolewski, J.J., Jiao, H.,Yi, T., Platanias, L.C. Association of the interferon-dependent tyrosine kinase Tyk2 with the hemotopoietic tyrosine phosphatase. J. Biol. Chem. 270: 18179-18182, 1995.

Yin, T., Shen, R., Feng, G.S., Yang, Y.C. Molecular characterization of specific interaction between SHP-2 phosphatase and Jak tyrosine kinases. J. Biol. Chem. 272: 1032-1037, 1997.

You M., Zhao Z.Z. Positive effects of SH2 domain-containing tyrosine phosphatase SHP-1 on epidermal growth factor- and interferon-gamma-stimulated activation of STAT transcription factors in HeLa cells. J. Biol. Chem. 272: 23376-23381, 1997.

You, M., Yu, D.H., Feng, G.S Shp-2 tyrosine phosphatase functions as a negative regulator of the interferon-stimulated Jak/STAT pathway Mol. Cell. Biol. 19: 2416-2424, 1999.

Yoshimura A., Ohkubo, T., Kiguchi, T., Jenkins, N.A., Gilbert, D.J., Copeland, N.G., Hara, T., Miyajima, A. A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. EMBO J. 14: 2816-2826, 1995.

Yu, C.-L. and Burakoff, S.J. Involvement of proteasomes in regulating Jak-STAT pathways upon interleukin-2 stimulation. J. Biol. Chem. 272: 14017-14020, 1997.

Zhang, J.-G., Metcalf, D., Rakar, S., Asimakis, M., Greenhalgh, C.J., Willson, T.A., Starr, R., Nicolson, S.E., Carter, W., Alexander, W.S., Hilton, D.J., Nicola, N.A. The SOCS box of suppressor of cytokine signaling-1 is important for inhibition of cytokine action in vivo. Proc. Natl. Acad. Sci. USA 98: 13261-13265, 2001.

Zhang, Y., Neo, S.Y., Han, J., Yaw, L.P., Lin, S.C. RGS16 attenuates galphaq-dependent p38 mitogen-activated protein kinase activation by platelet-activating factor. J. Biol. Chem. 274: 2851-2857, 1999.

Zhang, X., Blenis, J., Li, H.-C., Shindler, C., and Chen-Kiang, S. Requirement of serine phosphorylation for formation of STAT-promoter complexes. Science 267: 1990-1994, 1995.

Zhao, Y., Wagner, F., Frank, S.J., Kraft, A.S. The amino-terminal portion of the JAK2 protein kinase is necessary for binding and phosphorylation of the granulocyte-macrophage colony-stimulating factor receptor beta c chain. J. Biol. Chem. 270: 13814-12818, 1995.

Zhou, Y.J., Hanson, E.P., Chem, Y.Q., Magnuson, K., Chen, M., Swann, P.G., Wange RL, Changelian PS, O'Shea JJ. Distinct tyrosine phosphorylation sites in Jak3 kinase domain positively and negatively regulate its enzymatic activity. Proc. Natl. Acad. Sci. 94: 13850-13855, 1997.